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Arrest by Androgen Ablation and by Vitamin D3 Analog

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13. ABSTRACT (Maximum 200 Words)

Our data in LNCaP and *in vivo* in prostate cancers suggest that the cell cycle inhibitor, p27, is an important effector of growth arrest in the prostate. In the work of the last year, we identified that the cell cycle regulator, p27, mediates growth arrest by the vitamin D3 analog, EB1089. Work during the remainder of the grant period will address how processes regulating p27 are altered during prostate cancer progression. Effects of VDR activation and androgens on p27 function will be assayed. Experiments will clarify how phosphorylation affects p27 stability and its association with novel protein regulators and target cdks. We demonstrated the synergistic effect of physiologic concentrations of DHT and EB1089 to upregulate p27 and inhibit growth of prostate cancer cells. This work has led to our proposal to assay the effects of a combination of low dose DHT and EB1089 in pre-clinical trials using LNCaP xenografts in immunodeficient mice. If the synergistic effects of DHT and EB1089 to inhibit growth of prostate cancers or prevent tumor formation in nude mouse models are confirmed, these studies could lead to clinical trials in prostate cancer patients. Unraveling the pathways whereby these steroid hormones influence the cell cycle may define novel targets for anti-prostate cancer drugs. Moreover, our studies of p27 protein expression before and after NHT may provide a new marker to identify hormone resistant primary prostate cancers and stimulate development of novel treatment strategies.

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INTRODUCTION

Androgen interaction with the androgen receptor (AR) is important for prostate cancer cell proliferation (1, 2). The most effective therapy for prostate cancer is reduction of testosterone or of its most active metabolite, 5 α -dihydrotestosterone (DHT) by androgen ablation (3). However, disease progression to an androgen insensitive state severely limits treatment efficacy. Vitamin D3 suppresses prostate cancer growth in culture and in animals (4-8). However, growth inhibition by vitamin D3 is reduced in androgen independent prostate cancer lines, suggesting a connection between the actions of vitamin D3 and androgens on the cell proliferation cycle (8-10). In prostate cancer, oncogenic events may activate peptide growth factor signaling, leading to altered cross-talk with both AR and vitamin D3 receptor (VDR) pathways. There is very limited data on mechanisms whereby androgens or vitamin D3 affect the cell cycle in prostate cells. This proposal will examine how these two steroid hormones influence key cell cycle regulators and how these mechanisms are disrupted in prostate cancer progression. The cell cycle is governed by a family of cyclin dependent kinases (cdks) which are activated by cyclin binding and inhibited by cdk inhibitors (11). Passage through G1 phase is regulated by the activities of cyclin D- and cyclin E-associated cdks. The cdk inhibitor, p27, acts in early G1 to inhibit cyclin D1/cdk4 and cyclin E/cdk2 (12). Ubiquitin proteolysis regulates p27 levels and may be triggered by phosphorylation, by changes in associated proteins or by shifts in localization (13, 14). p27 increases during differentiation induced by both vitamin D3 (15, 16) and by high dose androgen (17). The loss of p27 in primary prostate cancers (18) and the observation that p27 knockout mice develop prostatic hyperplasia (19-21)(and A. Koff unpublished results), indicate that p27 is an important negative regulator of prostate epithelial proliferation. Further, our data in LNCaP suggest that p27 mediates G1 arrest by 100 nM DHT (see Oncogene reprint, Appendix 1).

The Hypothesis and Specific Aims of work proposed in this grant are summarized below:

We postulate that androgen ablation and vitamin D3 analogs induce prostate cell cycle arrest through inhibition of p27 phosphorylation, thereby inhibiting p27 degradation. Altered p27 regulation in prostate cancer may underlie resistance to cytostasis by androgen ablation. This will be pursued as follows. AIM 1. To identify common mechanisms whereby AR and VDR activation influence the cell cycle, we will compare effects of androgen, vitamin D3 analogs and AR blockade on cell cycle regulators in steroid sensitive and insensitive prostate lines. AIM 2. To test the role of p27 phosphorylation in G1 progression, we will assay whether proteasome inhibitors have the same effect as non-steroidal androgen receptor blockers on p27 levels. We will also mutate different p27 phosphorylation sites and test their effects on p27 stability, cdk binding and inhibitory activity. AIM 3. To determine whether p27 is required for growth inhibition by androgen blockade or by vitamin D3 analogs, we will use either dominant negative p27 or p27 antisense strategies in steroid sensitive prostate lines. AIM 4. We will test whether low levels of p27 in primary prostate cancers after pre-operative androgen ablation predict for androgen resistance in vivo.

PROGRESS REPORT BODY

The research accomplished on this grant during the period from January 1999 to January 2000 is reported below with specific reference to work proposed in the Statement of Work that accompanied the original grant application.

Task 1: Cell cycle effects of androgen, Vitamin D3 analogs and androgen receptor blockade in steroid sensitive and insensitive prostate.

a) How AR and VDR-mediated signals affect cell cycle regulators

- i) Cell cycle effectors of G1 arrest in LAPC-4^S and PCa 2 lines: Expression and activities of cyclins, cdks, cdk inhibitors and their complex formation will be assayed along with FACs analysis at intervals as asynchronous populations arrest after androgen changes of vit D3; **months 1-12**

Progress: The cell lines LAPC-4 and PCa-2 proved to be unsuitable for the studies proposed. The LAPC-4 line grew poorly in 2-dimensional culture and was not androgen dependent for growth. The PCa-2 line grew very slowly and was not useful for cell cycle studies. Thus all of the studies done so far have used the LNCaP prostate cancer cell line.

The assessment of the effects of high dose (100nM) dihydrotestosterone (DHT) on the cell cycle of LNCaP. The following summarizes our findings. Exposure to high dose DHT inhibits population growth of the human prostate carcinoma cell line, LNCaP. To determine the mechanism of growth arrest by high dose DHT, we assayed the changes in cell cycle profile and the cell cycle regulators that mediate these effects. Treatment of asynchronously growing LNCaP cells with 100nM DHT caused a G1 arrest. The proportion of cells in S phase fell from 20% to 2%, while the G1 fraction rose from 74% to 90% by 24 hours. Loss of phosphorylation of the retinoblastoma protein was noted and cdk4 and cyclin E/cdk2 activities fell. Inhibition of these G1 cyclin dependent kinases was not due to loss of either cyclin or cdk proteins nor to increases in the cdk inhibitors p16^{INK4A} and p21^{Cip1}. p21^{Cip1} protein levels remained constant, and cyclin E-associated p21^{Cip1} fell, suggesting that p21^{Cip1} is not relevant to this form of cyclin E/cdk2 inhibition. Of note, total p27^{Kip1} levels and cyclin E-associated p27^{Kip1} increased as cells arrested and the amount of the CAK activated cdk2 bound to cyclin E decreased. p27^{Kip1} immunodepletion experiments demonstrated that the DHT-mediated increase in p27^{Kip1} was sufficient to fully saturate and inhibit target cyclin E/cdk2. The inhibition of cyclin E/cdk2 by p27^{Kip1} contributes to G1 arrest of LNCaP following high dose DHT. p27^{Kip1} may be a key effector of androgen dependent growth modulation in prostate cancer cells. **This work was published in Oncogene (see Appendix 1).**

Effects of vitamin D3 analog EB1089 with or without low dose 3nM DHT on LNCaP cells.

Treatment of asynchronously growing LNCaP cells with 10⁻⁷ M EB1089 caused a gradual reduction in %S and an increase in the % cells in G1 (see Fig 1, Appendix 2). **All of the Figures 1-9 for this data are shown in Appendix 2.** Western analysis showed a progressive loss of cyclin A and B proteins and an increase in cdk inhibitor proteins p21, p27 and p16 (see Fig 2). This treatment had no effect on levels of cyclins E, D1, D2 nor on cdk2, cdk4 or cdk6 levels. There was a gradual loss of cyclin E- and cyclin D1-dependent kinase activities accompanying the G1 arrest of these cells (see Fig 3). IP/western analysis showed a progressive increase in the amount of cyclin E-bound p21

and p27 (see Fig 4 and 5), consistent with a role for both of these cdk inhibitors in inhibition of cyclin E-cdk2 complexes during the EB1089-induced arrest.

It had been demonstrated that the combination of Vitamin D3 and a physiologic dose of DHT could cause a synergistic growth arrest in prostate cancer cells (22). The vitamin D3 analogue EB1089 has the growth inhibiting properties of Vitamin D3 on prostate cancer cells, but does not cause hypercalcemia. EB1089 is currently in use in clinical trials as a neoadjuvant hormonal therapy prior to prostatectomy in prostate cancer patients in Canada. The EB1089 has few side effects and has been well tolerated by the men in these studies. To test whether the combination of EB1089 with low, physiologic dose DHT might be effective as an anti-prostate cancer therapy, we first assayed the effects of these drugs together on the cell cycle profile of LNCaP grown in tissue culture. The combination of EB1089 and DHT caused a much more rapid and complete G1 arrest in LNCaP than did EB1089 alone (see Fig 6). Low dose DHT(3nM) did not significantly affect the cell cycle on its own (not shown). The EB1089+DHT treated cells showed a more rapid and profound effect on losses of cyclin A and cyclin B1 proteins and on the increase of p27 (Fig 7). Again, levels of cyclin E protein and the cdk2 were not significantly changed by either drug combination. EB1089+ DHT caused a loss of cyclin D1 by 48 hours. There was little change in p21 levels in the first 48 hours of drug treatment although EB1089 caused a significant increase in p21 levels by 4-6 days post treatment. In Figs 8 and 9, it can be seen that the inhibition of cyclin E-cdk2 activity with EB1089+DHT was much more rapid over the 48 hours of treatment than that achieved with EB1089 alone. Cyclin E immune complexes showed a 5 fold increase in the amount of associated p27 in the EB1089+DHT treated cells. Our earlier work with LNCaP (see Appendix 1) suggests that the increase in p27 binding to cyclin E-cdk2 plays an important role in the G1 arrest of LNCaP cells treated with EB1089+DHT. We hope to demonstrate that loss of functional p27 through transduction of dominant negative mutant p27 protein leads to loss of EB1089+DHT-mediated G1 arrest. (see **Task 4** below). These experiments would definitively establish the essential role for p27 in this form of drug arrest and confirm that it is an appropriate target for the development of novel molecular based therapies for prostate cancer.

Because of the notable synergy of EB1089 and DHT in causing growth arrest of prostate cancer cells in tissue culture, we wish to extend our studies to an analysis of prostate cancer growth in an immunodeficient mouse model. We hope to test whether administration of EB1089 and implantation of DHT pellets in the experimental animals is more effective at inhibiting prostate cancer growth in vivo in nude mice than is EB1089 alone. Moreover, we also wish to assay whether the use of EB1089 in combination with low dose DHT (achieved by castration of the animals and implantation of DHT pellets which provide continuous stable physiologic DHT levels) is effective in preventing the development of prostate cancers when the LNCaP cells are injected subcutaneously in nude mice. When LNCaP is injected subcutaneously in a mixture of Matrigel in the CRI:nu/nu(CD-1)BR variant of nude mice, tumor take rates are over 95% at this institution. A copy of the Animal Use Protocol that has been approved by our institutional animal care committee will be submitted along with this progress report (**See Appendix 3**). All experiments will be carried out in conformity with the instructions in the "Guide for Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, revised 1985).

- ii) Do changes in p27 phosphorylation precede changes in p27 stability? ³⁵S methionine and ³²P-orthophosphate labeling and immunoprecipitation of p27 to determine if androgen or vitamin D3 analogs induce changes in both phosphorylation and half-life of p27; **months 12-24**

Progress: This work will be completed within the last year of this grant period as originally proposed

- iii) Relationship between p27 localization and stability: immunofluorescence and IHC of p27 assayed across cell cycle and following changes in androgen or vitamin D3 **months 6-18**

Progress: We have worked out the conditions for dual labeling of p27 and BrdU in cancer cells. These techniques will now allow us to monitor p27 levels and localization during cell cycle progression and during drug induced cell cycle arrest.

- b) Does altered post-translational regulation of p27 underlie androgen independence or vitamin D3 resistance in prostate cancer progression? months 12-30**

Steroid resistant variants of LNCAP and LCAP-4 cell lines will be grown, cell cycle synchronization methods will be developed (months 6-12)

Cell cycle regulators will be compared in sensitive and resistant cells using methods developed in a) above and p27 protein levels, phosphorylation and half-life and immunofluorescence studies of p27 localization will be compared in sensitive and resistant lines (months 10-30).

Progress: We have focused our attention on the characterization of the cell cycle effects of EB1089 and DHT in the LNCaP steroid sensitive cell line. During the next part of the grant period, our attention will be directed to the analysis cell cycle regulators in steroid independent variants of LNCaP and LAPC-4 prostate cancer cell lines.

Task 2: Does proteasome inhibition cause increased accumulation of phosphorylated p27?

- a) Do proteasome inhibitors have the same effect as non-steroidal AR blockers on p27?** use the chemical proteasome inhibitor, peptide-aldehyde N-acetyl-leuciny-leuciny-norleucinal-H (LLnL) to visualize the unstable forms of ubiquitin-bound p27 **months 12-18**

Progress: The proteasome inhibitor LlnL causes a marked increase in p27 levels due to inhibition of protein degradation and inhibits cell cycle progression from G1 to S phase.(not shown).

- b) Effects of phosphorylation defective p27 variants in prostate cancer cells?** Create p27 mutants by oligonucleotide directed mutagenesis using PCR. Test effects of mutant p27 in vitro and after transfection into LCAP-4 and assay effects on cell cycle and p27 stability; **months 18-30**

Progress: This work will be done in the second portion of this grant period as originally proposed.

Task 3: Is p27 essential for growth inhibition by vitamin D3 analogs or AR blockade ?

- a) Construction and testing of p27 antisense vectors; months 1-6**

b) Introduce p27^{k-VPKK} and/or anti-sense p27 into LCAP-4 and test effects on p27 expression by western and effects of loss of p27 on cell viability and on cycling; **months 12-24**

c) test if abrogation of p27 function causes resistance to vitamin D3 and AR blockade; **months 24-30**

Progress: During the first year of this grant period, we have worked extensively with p27 antisense oligonucleotides (p27AS oligos) from Gilead Scientific. Although these ASp27 oligos have been very effective in our hands in reducing p27 in the human breast cancer cells MCF-7, we have not been able to achieve sufficient reductions in p27 protein using these oligos in LNCaP to abrogate the increase in p27 binding to cyclin E-cdk2 that accompanies different forms of steroid induced cell cycle arrest. These results were published in the Oncogene paper in Appendix 1. We have abandoned the use of ASp27 oligos and plan to use vectors encoding a TATp27 fusion protein developed by Dr. Steve Dowdy's group (23). We have vectors encoding wild type p27 or a dominant negative mutant p27 (DNp27) protein fused to the Viral TAT sequence. The TAT sequence allows the direct transduction of DNp27 protein into prostate cancer cells. With this strategy, we hope to demonstrate that abolition of p27 functions confers resistance to growth arrest by vitamin D3 analogs and AR blockade. These studies will confirm the essential role of p27 for growth arrest by these different steroids in prostate cancer cells. These experiments will be completed within the remainder of the grant period and will be carried out as a collaboration with my former graduate student, who is currently an Assistant Professor at the University of Toronto, Urologic Oncologist, Dr. John Tsihlias.

Task 4: Do low levels of p27 in prostate cancers after pre-operative androgen ablation predict for androgen resistance in vivo?

Retrieval of 200 tumor blocks, up-dating of clinical patient data base, immunostaining for p27 and Mib-1, scoring of slides by pathologists and statistical analysis of clinical data and p27 and Mib1 scores: **months 1-24**

Progress: The patient hospital chart review has been completed for 49 patients with prostate cancer who received neo-adjuvant hormonal therapy prior to their prostatectomy at Sunnybrook Health Sciences Centre. Chart review has been completed for 125 patients who underwent resection of their prostate cancer without prior neo-adjuvant hormonal therapy. This chart review and collection of clinical data is required prior to the retrieval of blocks and the p27 immunohistochemistry (IHC) staining of biopsy and surgically removed tumor samples. The IHC staining of p27 and the analysis of whether p27 in the biopsy predicts the p27 staining in the tumor at surgical resection will be completed within the time frame of the grant. To complete the analysis of whether low p27 in the biopsy specimen can predict for failure of neoadjuvant hormonal therapy, the number of cases analyzed must be increased. These studies will not only confirm the importance of p27 as a prognostic factor for prostate cancer, they may also indicate that low p27 levels in the tumor biopsy specimen predict for hormone independence and poor outcome. This information would be of great value in making treatment decisions for patients with prostate cancer.

KEY RESEARCH ACCOMPLISHMENTS

1. Completed studies demonstrating that the G1 arrest of the LNCaP prostate cancer line by high dose dihydrotestosterone (DHT) is mediated by the cdk inhibitor p27 (see *Oncogene* reprint appended).
2. Showed that the vitamin D3 analog, EB1089, induces G1 arrest and demonstrated roles for p21 and p27 in this arrest.
3. Demonstrated that the vitamin D3 analog, EB1089 works synergistically with low, physiologic doses of DHT to mediate G1 cell cycle arrest. The two drugs work faster than EB1089 alone, due to a more rapid induction of p27 and increase its bind to cyclin E-cdk2 and an increase to binding to cyclin E-cdk2.
4. Established protocols for dual BrdU labeling and p27 staining by immunofluorescence in epithelial tumor cells.
5. Showed that p27 is stabilized and can be detected in the cytoplasm following treatment with the proteasome inhibitor LlnL.
6. Demonstrated that the use of antisense p27 oligonucleotides is not technically feasible in the LNCaP cell line. Although antisense p27 oligos prevented the increase in p27 levels following high dose DHT, the reduction in p27 protein was not sufficient to prevent its increases binding to cyclin E-cdk2 and G1 arrest by DHT in these cells (see *Oncogene* paper appended).
7. Obtained clones for direct transduction of 27 protein into prostate cancer cells using a TAT fusion vector. The strategy will allow us to directly transduce prostate cancer cells with a dominant negative p27 protein. This strategy may allow us to ascertain whether loss of p27 function can abrogate growth arrest by vitamin D3 analogues and low dose DHT.
8. Re Aim 4 of the Grant: Chart review has been completed for 49 patients with prostate cancer who received neo-adjuvant hormonal therapy prior to their prostatic resection. Chart review has been completed for 125 patients who underwent resection of their prostate cancer without prior neo-adjuvant hormonal therapy. This chart review is required prior to the retrieval of blocks and the p27 immunohistochemistry staining of biopsy and surgically removed tumor samples.

REPORTABLE OUTCOMES

Manuscript

Tsihlias J, Zhang W, Bhattacharya N, Flanagan M, Klotz L, Slingerland J. Involvement of p27^{Kip1} in G1 arrest by high dose 5 α -dihydrotestosterone in LNCaP human prostate cancer cells. *Oncogene* 19:670-679, 2000. (see Appendix 1)

CONCLUSION AND FUTURE DIRECTIONS

The molecular basis of growth inhibition by androgen ablation is poorly understood. Our data in LNCaP and *in vivo* in prostate cancers (18) suggest that p27 is an important effector of growth arrest in the prostate. In the work of the last year, we have identified that the cell cycle regulator, p27, mediates growth arrest by high dose DHT (Oncogene paper appended) and also by the vitamin D3 analog, EB1089. Work during the remainder of the grant period will address how the processes regulating p27 are altered during progression to androgen independent prostate cancer. Effects of VDR activation and androgens on p27 function will be assayed. Experiments proposed aim to clarify how phosphorylation affects p27 stability and its association with novel protein regulators and target cdks. The cloning and characterization of novel p27 regulators would form the basis for future projects in my laboratory.

We have demonstrated the synergistic effect of low, physiologic concentrations of DHT and EB1089 in growth inhibition of prostate cancer cells. This work has led to our proposal to assay the effects of a combination of low dose DHT and EB1089 in pre-clinical trials using LNCaP xenografts in immunodeficient mice. If the synergistic effects of DHT and EB1089 to inhibit growth of prostate cancers or prevent tumor formation in nude mouse models are confirmed, these studies could lead fairly soon to clinical trials in prostate cancer patients. EB1089 is already being used in clinical trials in Canada in the neo-adjuvant setting prior to prostate cancer surgery.

Unraveling the pathways whereby these steroid hormones influence the cell cycle may define novel targets for anti-prostate cancer drugs. Moreover, our studies of p27 protein expression before and after NHT may provide a new marker to identify hormone resistant primary prostate cancers and stimulate development of novel treatment strategies.

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APPENDICES

1. Tsihlias J, Zhang W, Bhattacharya N, Flanagan M, Klotz L, Slingerland J. Involvement of p27^{Kip1} in G1 arrest by high dose 5 α -dihydrotestosterone in LNCaP human prostate cancer cells. *Oncogene* 19:670-679, 2000.
2. Figures 1- 9
3. Animal Use Protocol #99-029 "In vivo assays of growth suppression of prostate cancer xenografts by vitamin D3 analogs and dihydrotestosterone" and Appendix 6 re: Research Involving Animals; letters from NIH.
4. Revised Year 2 Budget

Involvement of p27^{Kip1} in G1 arrest by high dose 5 α -dihydrotestosterone in LNCaP human prostate cancer cells

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The cell cycle is governed by cyclin dependent kinases (cdks), which are activated by binding of cyclins, inhibited by cdk inhibitors and regulated by phosphorylation and dephosphorylation. Exposure to high dose dihydrotestosterone (DHT) inhibits population growth of the human prostate carcinoma cell line, LNCaP. To determine the mechanism of growth arrest by high dose DHT, we assayed the changes in cell cycle profile and the cell cycle regulators that mediate these effects. Treatment of asynchronously growing LNCaP cells with 100 nM DHT caused a G1 arrest. The proportion of cells in S phase fell from 22 to 2%, while the G1 fraction rose from 74 to 92% by 24 h. Loss of phosphorylation of the retinoblastoma protein was noted and cdk4 and cyclin E/cdk2 activities fell. Inhibition of these G1 cyclin dependent kinases was not due to loss of either cyclin or cdk proteins nor to increases in the cdk inhibitors p16^{INK4A} and p21^{Cip1}. p21^{Cip1} protein levels remained constant, and cyclin E-associated p21^{Cip1} fell, suggesting that p21^{Cip1} is not relevant to this form of cyclin E/cdk2 inhibition. Of note, total p27^{Kip1} levels and cyclin E-associated p27^{Kip1} increased as cells arrested and the amount of the CAK activated cdk2 bound to cyclin E decreased. p27^{Kip1} immunodepletion experiments demonstrated that the DHT-mediated increase in p27^{Kip1} was sufficient to fully saturate and inhibit target cyclin E/cdk2. The inhibition of cyclin E/cdk2 by p27^{Kip1} contributes to G1 arrest of LNCaP following high dose DHT. p27^{Kip1} may be a key effector of androgen dependent growth modulation in prostate cancer cells. *Oncogene* (2000) 19, 670–679.

Keywords: p27; cell cycle; prostate cancer; androgen; cyclin E/cdk2

Introduction

Androgen interaction with the androgen receptor (AR) is important for growth and development of both the normal prostate and of prostate cancer (Hakimi *et al.*, 1996). At present, the most effective therapy for prostate cancer is reduction of testosterone or of its most active metabolite, 5 α -dihydrotestosterone (DHT)

by different treatments collectively referred to as androgen ablation (Catalona, 1994). However, tumor progression to an androgen insensitive state severely limits the efficacy of these treatments. We have investigated how this steroid pathway influences key cell cycle regulators in the androgen sensitive, human prostate cancer cell line, LNCaP. An understanding of how cell cycle progression is influenced by androgen pathways, and how these mechanisms are disrupted in prostate cancer progression, may lead ultimately to new methods of achieving cytostasis in hormone resistant prostate cancers.

Progression through the cell cycle is governed by a family of cyclin dependent kinases (cdks), whose activity is regulated by phosphorylation (Solomon, 1993), activated by binding of cyclins (Morgan, 1995; Sherr, 1994) and inhibited by the cdk inhibitors (Reed *et al.*, 1994; Sherr and Roberts, 1995). The cdks regulate biochemical pathways, or checkpoints, which integrate mitogenic and growth inhibitory signals, monitor chromosome integrity, and coordinate cell cycle transitions (Hartwell, 1992; Murray, 1992). Passage through G1 into S phase is regulated by the activities of cyclin D-, cyclin E-, and cyclin A-associated kinases. Cyclin B-dependent kinases regulate the G2/M transition.

Two families of cdk inhibitors mediate cell cycle arrest following growth inhibitory stimuli (Sherr and Roberts, 1995, 1999). The inhibitor of cdk4, INK4, family members p15^{INK4B}, p16^{INK4A}, p18^{INK4C}, and p19^{INK4D} bind cdk4 and cdk6 specifically and inhibit cyclin D binding. Members of the KIP or kinase inhibitor protein family, which include p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}, bind and inhibit target cyclin/cdk complexes. The cdk inhibitor, p27, acts during G0 and the early G1 phase of the cell cycle to inhibit G1 cyclin/cdk complexes (Polyak *et al.*, 1994a,b; Toyoshima and Hunter, 1994; Slingerland *et al.*, 1994; Hengst *et al.*, 1994). In many cell types, p27 is essential for quiescence. Antisense p27 inhibits exit from the cell cycle following serum withdrawal (Coats *et al.*, 1996; Rivard *et al.*, 1996) and antisense p27 stimulates estradiol depleted quiescent MCF-7 breast cancer cells to re-enter cell cycle (Cariou, Donovan and Slingerland, in preparation). p27 levels are regulated by post-transcriptional mechanisms including both translation and proteolysis (Hengst and Reed, 1996; Pagano *et al.*, 1995; Millard *et al.*, 1997). In human cell lines, phosphorylation of p27 is maximal in G1, just prior to the drop in p27 protein that occurs at S phase (Pagano *et al.*, 1996). Phosphorylation of p27 triggers its proteolytic degradation (Vlach *et al.*, 1997; Sheaff *et*

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et al., 1997; Montagnoli *et al.*, 1999; Tsvetkov *et al.*, 1999), as is the case for the yeast cdk inhibitor Sic1 (Schneider *et al.*, 1996; Tyers, 1996).

p27 increases during differentiation in many cell types, including differentiation induced by vitamin D3 (Hengst and Reed, 1996; Wang *et al.*, 1996) and by androgen in the prostatic epithelium (Chen *et al.*, 1996). p27 knockout cells manifest altered differentiation programs (Casaccia-Bonnel *et al.*, 1997). Indeed, p27 knockout mice develop multiple organ hyperplasia (Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996), including prostatic hyperplasia (Cordon-Cardo *et al.*, 1998), underlining the importance of p27 as an inhibitor of prostate cell proliferation.

Increasing evidence suggests that the cyclins, cdks and cdk inhibitors are either themselves targets for genetic change in cancer or are disrupted secondarily by other oncogenic events (Hunter and Pines, 1994). Since they oppose mitogenic stimuli, the cdk inhibitors are good candidates as tumor suppressors. Although genetic changes in the *p16* gene support a tumor suppressor (TS) role for this inhibitor in cancers (Bates and Peters, 1995), mutations in *p27* have been identified only rarely (Kawamata *et al.*, 1995; Pietersen *et al.*, 1995; Ponce-Castaneda *et al.*, 1995). A reduction in p27 protein could contribute to resistance to growth inhibitory factors, deregulation of cell proliferation, and oncogenic change. Although normal human epithelia of the breast, prostate, lung, and colon express high levels of p27 protein, this protein is frequently reduced in primary carcinomas at these sites (reviewed in Cariou *et al.*, 1998). The reduction of p27 protein is of prognostic importance in these cancers and correlates with an aggressive tumor phenotype *in vivo* (Catzavelos *et al.*, 1997; Loda *et al.*, 1997; Porter *et al.*, 1997; Tan *et al.*, 1997; Mori *et al.*, 1997; Esposito *et al.*, 1997; Tsihlias *et al.*, 1998). Enhanced proteolytic degradation may underlie the loss of p27 in tumor cells (Catzavelos *et al.*, 1997; Loda *et al.*, 1997; Esposito *et al.*, 1997). Thus, changes in the post-translational mechanisms which target p27 for degradation, may be germane to oncogenesis and/or tumor progression.

In a study of p27 immunostaining in primary prostate cancer, we found that loss of p27 protein was an independent prognostic factor, predicting reduced time to recurrence following radical prostatectomy ($P=0.047$, risk ratio 2.08) (Tsihlias *et al.*, 1998). In a small subset of patients, androgen ablation therapy prior to tumor removal appeared to increase p27 protein, raising the possibility that androgens may modulate p27 degradation pathways *in vivo*. In the present study, the effect of high dose androgen on cell cycle progression was investigated in a model of human prostate cancer, the LNCaP cell line.

LNCaP is an androgen-sensitive human prostate cancer cell line derived from a metastasis to a supraclavicular lymph node (Horoszewicz *et al.*, 1983). The line has an aneuploid human male karyotype, and is tumorigenic when implanted into nude mice. The potent androgen, 5 α -dihydrotestosterone (DHT), modulates LNCaP proliferation in a well-characterized fashion. DHT stimulates proliferation at concentrations between 0.1 and 1.0 nM DHT and proliferation is inhibited at DHT concentrations below 0.1 nM or above 10 nM DHT. Androgens have also been shown to mediate biphasic growth response in the prostate *in vivo*. Low

dose androgen administered to castrate rats will reverse prostate atrophy and induce proliferation in the prostate gland. Higher doses of androgen cause growth arrest by inducing differentiation of prostate epithelial cells (Chen *et al.*, 1996). Because our study of primary tumors suggested that p27 regulation may be importantly altered in human prostate cancers, we wished to explore further how androgen may modulate the cell cycle and p27 function in the prostate cancer line, LNCaP.

Results

100 nM dihydrotestosterone causes G1 arrest of LNCaP

Treatment of asynchronously growing LNCaP cells with 100 nM DHT for 24 h induced a G1 arrest. The proportion of cells in G1 went from 70%, in the asynchronously growing untreated cells, to 92% at

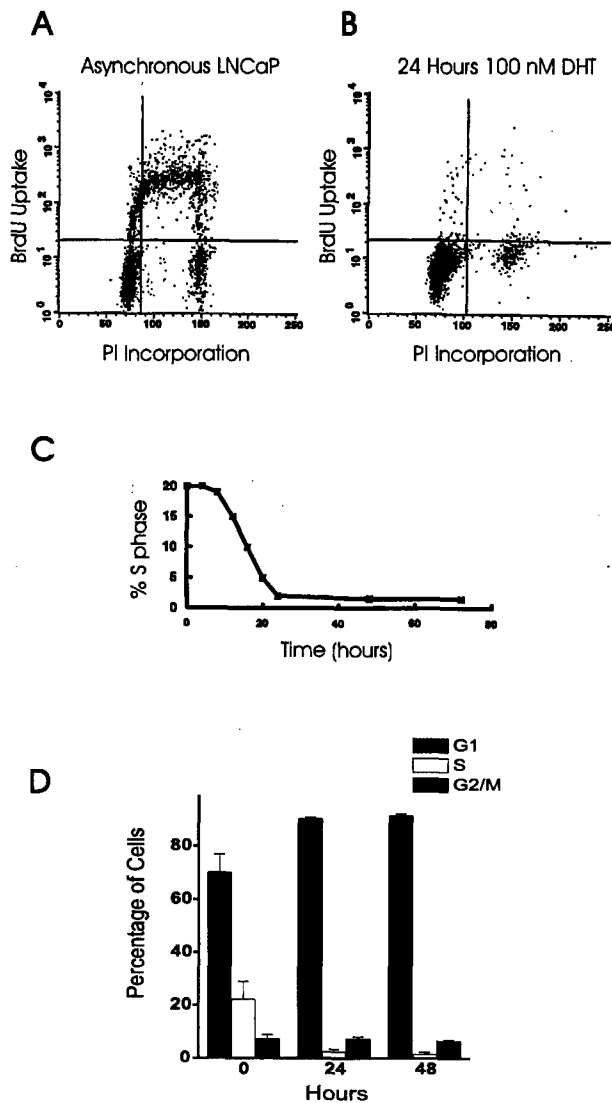


Figure 1 FACS analysis of LNCaP treated with 100 nM dihydrotestosterone. (a) Dot plot showing relative uptake of BrdU and PI in asynchronously growing LNCaP. (b) Decreased BrdU uptake indicating arrest of DNA synthesis after 24 h treatment with 100 nM DHT. (c and d) Cells were harvested and prepared for FACS analysis at various intervals after addition of 100 nM DHT to culture media. G1 arrest was maximal at 24 h. Results shown in (d) are the average of three separate experiments

maximal arrest by 48 h. Cells in S phase fell from 22% of the untreated population to 2% within 24 h (Figure 1). A sub-G1 population was not observed. No significant growth arrest was observed when cells were cultured in media supplemented with 1 and 10 nM DHT (data not shown).

The cell cycle arrest persisted for up to 96 h in media supplemented with DHT. This DHT-mediated cell cycle arrest was not accompanied by cytotoxicity, based on morphological appearance and the use of Trypan blue exclusion staining. The proportion of cells that failed to exclude Trypan blue was similar in treated and untreated cells. Treatment of LNCaP cells with 50 nM DHT also caused a G1 arrest within 48 h. To determine whether high dose DHT was inducing a form of irreversible differentiation, cells were cultured for 48 h in 50 and 100 nM DHT and then transferred to media without additional DHT for a further 24 h. Upon removal of supplemental DHT from the culture media, cells underwent a prompt release from G1 arrest, showing an asynchronous cell cycle profile within 24 h (S phase fraction 20%) (Table 1).

To determine whether the effects of 100 nM DHT on prostate cancer cell proliferation could occur in the absence of the AR and AR signaling, two steroid-independent prostate cancer lines, PC-3 (Kaighn *et al.*, 1979) and DU145 (Stone *et al.*, 1978) were treated with 100 nM DHT. Neither the PC-3 nor the DU145 prostate cancer cell lines express detectable AR (Kaighn *et al.*, 1979; Stone *et al.*, 1978). The addition of 100 nM DHT to the culture medium had no effect on either PC-3 or DU145 cell proliferation (data not shown). Treatment of LNCaP cells with 100 nM DHT for 48 h led to an increased secretion of prostate specific antigen as determined by Hybritech assay on culture media. PSA concentration (ng/ml) in conditioned media from cells with/without 100 nM DHT was 383.5/167.5 and 443.1/340.3 on two separate occasions. Although the results were not normalized for cell number, cells were initially plated equally. After 48 h, the number of cells on DHT treated plates would be less than that on untreated plates. Thus, relative cellular production of secreted PSA was increased by exposure to 100 nM DHT. A Western blot did not demonstrate any difference in cellular PSA levels as cells underwent arrest (data not shown).

DHT mediated G1 arrest is accompanied by accumulation of nuclear p27 protein

Immunohistochemistry performed on asynchronous populations of LNCaP cells demonstrated a heterogeneous nuclear immunoreactivity for p27. Cells

arrested in G1 after treatment with 100 nM DHT for 48 h demonstrated uniformly strong nuclear staining for p27 protein (Figure 2). Controls reacted with secondary antibody alone did not show any significant nuclear staining. Pre-adsorption of the primary antibody with blocking peptide abolished the nuclear staining (data not shown).

Western analysis at intervals after treatment with 100 nM DHT showed loss of phosphorylation of the retinoblastoma protein (pRb) within 16 h, with predominantly hypophosphorylated pRb detected at 48 h. There was no appreciable change in the protein levels of cdk2, cdk4, and cdk6, cyclins D1 and E, or the cdk inhibitors p16^{INK4A} and p21^{Cip1}. Levels of cyclins A and B1 fell significantly. This is consistent with the DHT-mediated inhibition of entrance into S and G2/M phases, where peak expression of these latter two proteins is known to occur. An increase in p27 protein levels was appreciable within 4 h after addition of DHT, with peak levels fourfold above baseline detected by 48 h, as determined by densitometry (Figure 3).

The increase in p27 saturates and inhibits cyclin E/cdk2 in DHT-arrested LNCaP

Inhibition of cyclin E/cdk2 activity was notable within 12 h after the addition of 100 nM DHT to the asynchronously growing cells, with minimal activity detected at 24 h (Figure 4b). To further investigate the mechanism of inhibition of cyclin E/cdk2, these complexes were examined by immunoprecipitation of cyclin E followed by Western blotting to detect associated proteins. No dissociation of cyclin E/cdk2 complexes was observed, however, the amount of CAK-activated threonine 160 phosphorylated cdk2 bound to cyclin E decreased. Phosphorylation by cdk activating kinase (CAK) shifts cdk2 to its faster mobility form on SDS-PAGE (Gu *et al.*, 1992). The amount of cyclin E-associated p21 fell, suggesting that p21 is not relevant to this form of cyclin E/cdk2 inhibition. The amount of cyclin E-associated p27 increased 3–4-fold, by densitometry, as the cells entered G1 arrest (Figure 4a). In other cellular contexts, such an increase in p27 has been shown to saturate cellular cyclin E/cdk2 (Reynisdottir *et al.*, 1995).

To determine whether this increase in p27 was sufficient to saturate target cyclin E/cdk2 and induce cell cycle arrest, p27 was immunodepleted by three serial immunoprecipitations from both asynchronously growing cell lysates and from DHT-arrested cell lysates. Cyclin E immune complexes were examined before and after p27-immunodepletion. p27 immunodepletion caused a significant reduction of cyclin E protein from asynchronously growing cell lysates (Figure 4c). This reflects the substantial proportion of G1 phase cells in an asynchronous population of LNCaP. In spite of a significant reduction in the level of cyclin E, the remaining cyclin E immune complexes showed almost the same kinase activity after p27 immunodepletion as before (Figure 4c, left panel). This is consistent with most p27-bound cyclin E complexes having minimal kinase activity. There was virtually no detectable cyclin E remaining after p27 immunodepletion of DHT-arrested cell lysates. Thus, the increase in p27 by DHT treatment was sufficient to saturate and inhibit the cellular cyclin E/cdk2 complexes.

Table 1 Cell cycle profiles of LNCaP

Culture conditions	%G1	%S	%G2/M
Asynchronously growing cells	76	19	5
100 nM DHT 48 h	93	2	5
100 nM DHT 48 h then no DHT 24 h	74	19	7
50 nM DHT 48 h	93	2	5
50 nM DHT 48 h then no DHT 24 h	72	20	8

Asynchronously growing cells were cultured in RPMI 1640 plus 5% FBS with either 50 or 100 nM 5 α -dihydrotestosterone (DHT) for 48 h. Cells were then either collected for FACS analysis or washed and fresh media without supplemental DHT was added to plates for a further 24 h prior to FACS analysis

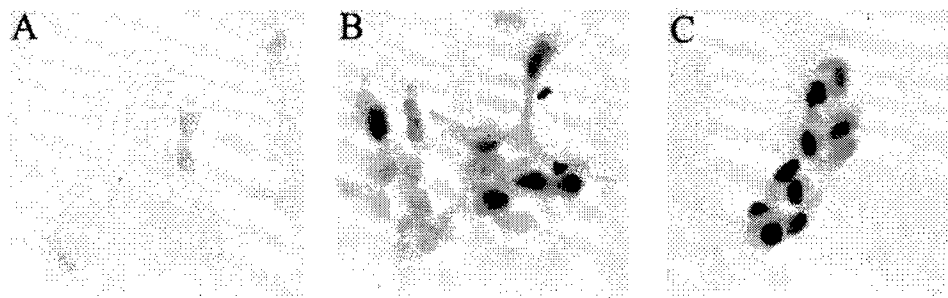


Figure 2 p27 nuclear staining of LNCaP cells in tissue culture. Cells were cultured on poly-L-lysine coated glass slides, fixed and stained for p27 as described in the Materials and methods section. (a) Control sample obtained by staining with secondary antibody alone. (b) Asynchronously growing cells. (c) Cells arrested by treatment with 100 nM DHT show uniform strong nuclear p27 staining

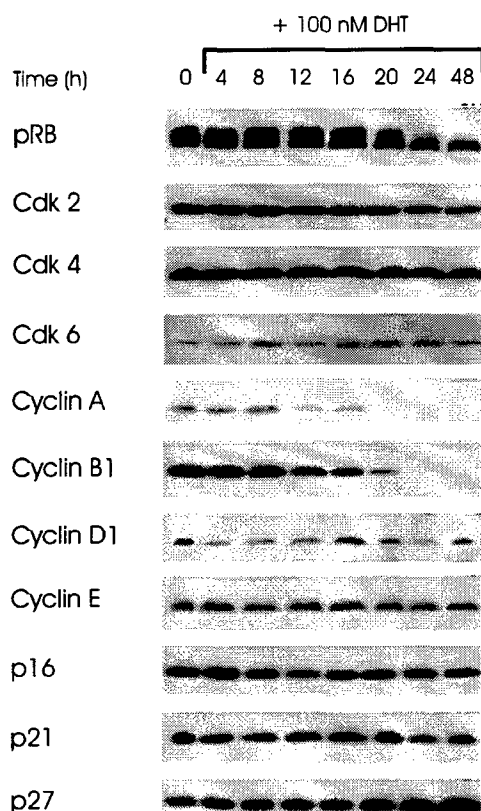


Figure 3 Cell cycle regulators during G1 arrest by DHT. Cells were harvested at indicated time intervals after addition of 100 nM DHT to culture media. Lysates were prepared and resolved using 17.5% SDS-PAGE. Proteins were detected by immunoblotting with the indicated antibodies

Antisense p27 failed to prevent the increased binding of p27 to cyclin E after high dose DHT

Antisense p27 oligonucleotides (ASp27) were transfected into asynchronously growing LNCaP cells. Six hours post transfection, 100 nM DHT was added to control (lipid alone), mismatch oligonucleotide (MSp27) and ASp27 transfected cells. In both oligonucleotide-transfected and non-transfected controls, 100 nM DHT induced G1 arrest. MSp27 and control cells showed the same increase in p27 by DHT as did LNCaP treated with 100 nM DHT alone. ASp27 prevented the fourfold rise in p27 protein following DHT treatment. p27 levels in the cells treated with

ASp27 and DHT together were similar to untreated asynchronously growing LNCaP. However, when cyclin E immune complexes were analysed, ASp27 treated cells still showed an increase in the binding of p27 to cyclin E complexes following 100 nM DHT, in spite of the modest reduction in total p27 levels. ASp27 treatment did not reduce total p27 protein levels to a sufficient degree in LNCaP to prevent the DHT-mediated increased association of p27 with its target kinase cyclin E/cdk2 (data not shown).

Inhibition of cyclin D-bound cdk4 by high dose DHT

The cyclin D-associated kinases also function in G1 to regulate the transition into S phase. To assay the effects of 100 nM DHT on these complexes, cdk4 was immunoprecipitated and kinase activity assayed at various intervals during the arrest of the LNCaP cells. Cdk4 activity was strongly inhibited by treatment with 100 nM DHT (Figure 5b). Immunoprecipitation of cdk4 and its associated complexes did not demonstrate any dissociation of cyclin D1 from cdk4. The level of cdk4-bound p16 remained unchanged. There was a rise in p15 association with cdk4 between 0–12 h after addition of DHT, which persisted to 24 h. Between 24–48 h, there was no further reduction in the per cent S phase cells, and cdk4-bound p15 actually decreased slightly by 48 h, possibly reflecting progression into a quiescent state. The levels of p21 and p27 bound to cdk4 decreased as cells underwent arrest.

Discussion

The LNCaP cell line is the most commonly studied model of androgen-sensitive prostate cancer. This cell line shows a characteristic bell shaped growth curve in response to increasing concentrations of androgen in the growth media (Horoszewicz *et al.*, 1983; Lee *et al.*, 1995; Kim *et al.*, 1996). Although the dose-dependent differences in the effects androgen on cell numbers have been known for nearly two decades, the mechanism of the growth inhibition by high dose androgen was poorly understood. Androgens are required for normal growth and differentiation in prostate epithelial cells. Chen *et al.* (1996) studied the effects of androgen on the cell cycle of normal prostate *in vivo* in rats. Castration resulted in atrophy of the gland due to

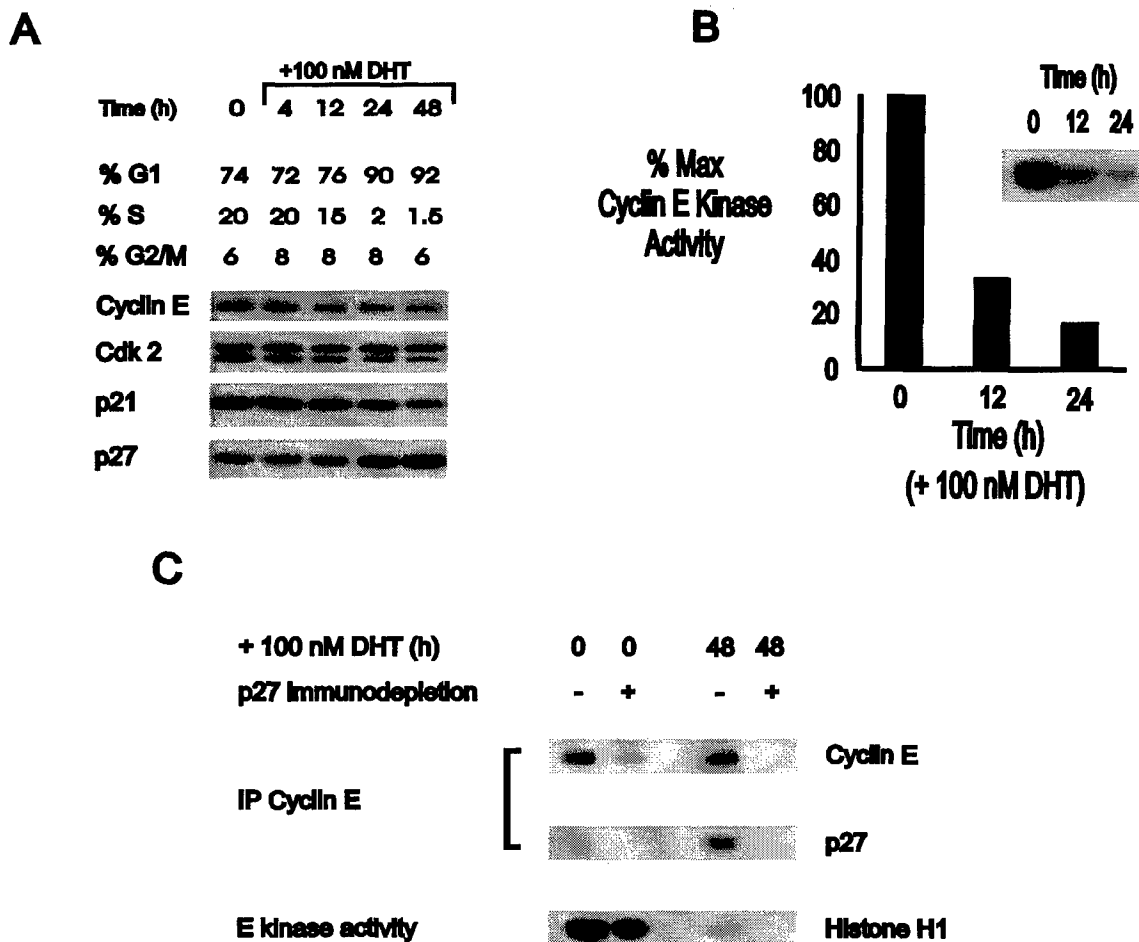


Figure 4 p27 saturates cyclin E/cdk2 complexes and inhibits this kinase following treatment of asynchronously growing LNCaP with 100 nM DHT. (a) Cyclin E immune complexes. Cells were recovered at intervals after addition of DHT to culture media. DNA profiles were determined by FACS analysis, and the percentage of cells in G1, S, and G2/M is indicated for each time point. Cyclin E was immunoprecipitated, complexes resolved by SDS-PAGE and immunoblots were reacted with antibodies to detect associated proteins. (b) Cyclin E/cdk2 Kinase Activity. Cyclin E was immunoprecipitated from lysates collected in (a) above and associated kinase activities were assayed. Reaction products were resolved by SDS-PAGE, and gels were dried and exposed to film. Radioactivity in the Histone H1 substrate was quantitated by PhosphorImager and expressed as a percentage of maximum activity after subtraction of background from control immunoprecipitates. (c) p27 immunodepletion experiments. p27 was serially immunodepleted from lysates collected at either 0 or 48 h after addition of 100 nM DHT to media. Cyclin E-associated proteins and cyclin E-associated kinase activities were assayed before and after p27 immunodepletion from the T=0 and T=48 h samples

apoptosis and arrest of surviving cells. Re-administration of testosterone induced transient epithelial cell proliferation followed by decreased cellular proliferation and an increase in p27. It was postulated that the increased p27 was playing a role to inhibit proliferation as regenerating prostatic tissue underwent steroid-mediated differentiation. The paradoxical effects of androgen stimulation in LNCaP (growth stimulation at low concentrations of androgen, growth inhibition with increased PSA secretion at high concentrations) may represent a normal physiological mechanism that has been retained by this tumor cell line.

Several lines of evidence suggest that the G1 arrest by high dose DHT is an AR mediated effect. In our study, as in others (Lee *et al.*, 1995), high dose DHT induced an increase in PSA secretion by the LNCaP cells. PSA is a serine protease that is normally secreted by the prostatic epithelium. We demonstrated higher PSA production in conditioned media from cells arrested after 48 h in 100 nM DHT than in cells without added androgen. This result and our observation that cellular PSA levels were not increased by

Western analysis are consistent with a DHT-induced increase in secretion of PSA. Treatment of AR-negative PC-3 cells and DU145 cells with 100 nM DHT affected neither cellular proliferation nor PSA secretion (not shown). Furthermore, PC-3 cells that have been genetically engineered to express the AR show growth arrest in response to androgen treatment (E Brown, personal communication).

The present study demonstrates that the decreased rate of growth for LNCaP at high doses of DHT is due to G1 arrest and not to cell death. Growth inhibition with G1 arrest was demonstrated at doses of 50 and 100 nM DHT, whereas, no significant growth arrest was observed with doses between 1–10 nM DHT. We saw no morphological evidence of cell toxicity and Trypan blue exclusion did not differ between treated and untreated cells. Furthermore, the G1 arrest appeared fully reversible on removal of the high dose DHT at 48 h. The longest treatment interval was 96 h, with maximal arrest noted at 24 h. Whether exposure to 100 nM DHT longer than 96 h might induce an irreversible G1 arrest is not known.

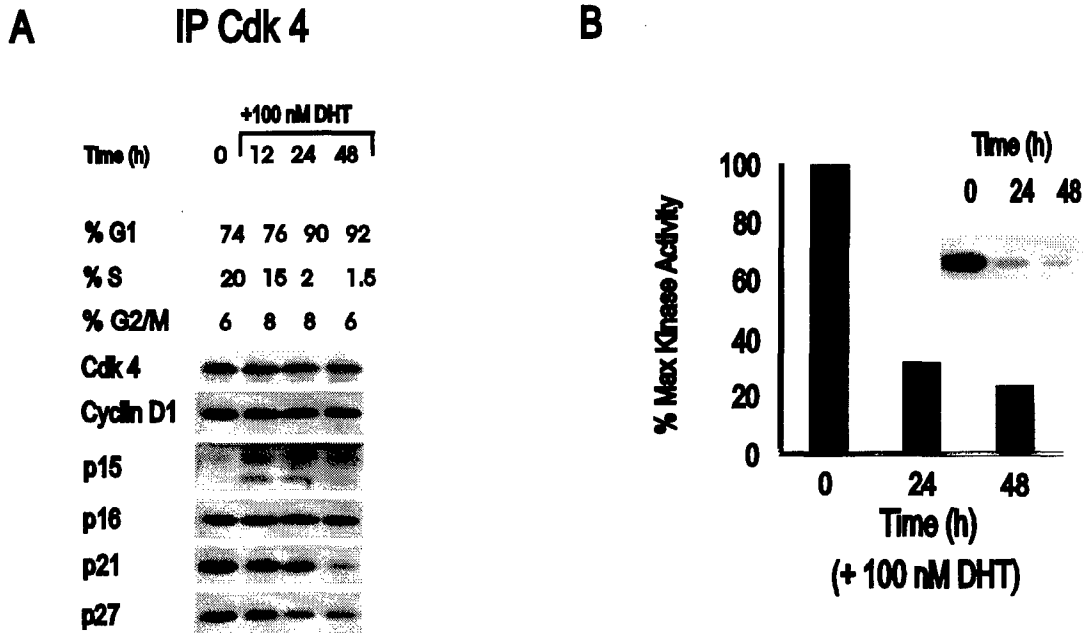


Figure 5 Inhibition of Cdk4 activity during DHT-mediated G₁ arrest. Asynchronous LNCaP cultures were treated with 100 nM DHT and cells were recovered for FACS analysis and preparation of protein lysates. (a) Cdk4 complexes. Cdk4 was immunoprecipitated and complexes were resolved and immunoblotted as for Figure 4a. (b) Cdk4 kinase assay. Cdk4 was immunoprecipitated and kinase activity was assayed as in Materials and methods

High dose DHT caused a loss of pRB phosphorylation as has been observed in other forms of G₁ arrest (Laiho *et al.*, 1990; Sandhu *et al.*, 1997). Hypophosphorylated pRB binds and inactivates E2F, thereby inhibiting the function of that transcription factor, and precluding transcription of genes required for S phase entry (DeGregori *et al.*, 1995). Phosphorylation of pRB leads to its dissociation from E2F, and to E2F activation (Chellappan *et al.*, 1991). The *pRB* gene is wild type in LNCaP (Peehl, 1994). Since both cyclin D-associated kinases and cyclin E/cdk2 activities contribute to pRB phosphorylation (Sherr and Roberts, 1995), the effects of DHT on these cyclin/cdks were assayed.

Cyclin E-dependent kinase activity fell as cells underwent arrest. This was accompanied by a fourfold overall increase in p27 protein. More importantly, the increase in p27 protein was sufficient to fully saturate cyclin E/cdk2 complexes. p27-immunodepletion demonstrated that essentially all of the cellular cyclin E was bound to p27 in these DHT-arrested cells. This supports the conclusion that the increase in p27 contributes causally to cyclin E/cdk2 inhibition and to the G₁ arrest observed. In other systems, a similar increase in p27 levels has been shown to be sufficient to saturate and inactivate cyclin E/cdk2 (Reynisdottir *et al.*, 1995). The fact that cyclin E-associated p21 fell as cells arrested, suggests that p21 is not likely to be involved in inhibition of this kinase.

ASp27 treatment prevented the fourfold increase in p27 levels following exposure to 100 nM DHT. However, ASp27 failed to prevent DHT-mediated G₁ arrest. In spite of the modest reduction in p27 protein levels, the increased association of p27 with cyclin E/cdk2 complexes was intact in ASp27 treated cells arrested by 100 nM DHT. This suggests that factors that lead to an increased affinity of binding of p27 to

cyclin E/cdk2 following DHT treatment may be more important to the inhibition of this kinase than the increase in p27 protein levels alone.

Cdk activating kinase (CAK) mediated phosphorylation of cdk2 on threonine 160 is required for catalytic activity (Gu *et al.*, 1992). In this study, cyclin E-bound cdk2 showed a progressive loss of CAK activation, with accumulation of the slower mobility form of cyclin E-bound cdk2. Kato *et al.* (1994) have demonstrated that p27 can inhibit CAK activation of cyclin D-associated cdk4 during cyclic AMP mediated G₁ arrest in macrophages. Increased binding of p27 to cyclin E/cdk2 may modulate the conformation of these complexes and block the access of CAK to its catalytic sites on cdk2, thereby preventing the activating phosphorylation of cdk2. A similar loss of CAK activation of cyclin E-bound cdk2 has been demonstrated in TGF- β arrested cells (Koff *et al.*, 1993; Slingerland *et al.*, 1994).

Cdk4 kinase activity was also inhibited by 100 nM DHT. p16, p21, and p27 are not likely involved in the inhibition of this kinase, since p16 association with cdk4 remained constant and dissociation of p21 and p27 from cdk4 was observed. p15 binding to cdk4 was increased at 12 h and remained elevated until 24 h. The accumulation of p15 may facilitate dissociation of p21 and p27 from cdk4 complexes. Versions of this model of action for p15 have been suggested previously in G₁ arrest due to TGF- β (Reynisdottir *et al.*, 1995; Sandhu *et al.*, 1997). It is also possible that the affinity of p27 for cyclin E/cdk2 is actively regulated as suggested by Sheaff *et al.* (1997), and this may be independent of the effects of p15 on cyclin D/cdk4 complexes (Sandhu *et al.*, 1997).

The INK4 family of cdk inhibitors have been shown to inactivate their target kinases by binding to them and displacing the associated cyclin (Parry *et al.*, 1995;

Sandhu *et al.*, 1997). Although we did not see cyclin D1 dissociation from cdk4 as kinase activity diminished, it is possible that p15 may have displaced cyclin D2 or D3. However, we were unable to detect cyclins D2 or D3 in cdk4 complexes using commercially available antibodies. Clearly, there may be more than one mechanism contributing to DHT-mediated G1 arrest. Although p27 appears to be important in the inhibition of cyclin E/cdk2, other mechanisms, including p15, may act to inhibit the D-type cyclin/cdks.

Since 1941, when Huggins and Hodges discovered that prostate cancer undergoes regression in response to castration, there have been few major advances in the management of metastatic prostate cancer (Huggins *et al.*, 1941). Leutinizng hormone releasing hormone (LHRH) analogs that induce castrate levels of testosterone have obviated the need for castration (Peeling, 1989). In spite of advances in the hormonal therapy of prostate cancers, progression to a hormone independent state invariably occurs. Thus, there is clearly a need for development of novel treatment approaches for advanced prostate cancer. A greater understanding of how cell cycle regulators respond to androgenic stimuli in normal and malignant prostatic epithelial cells could lead to the identification of new targets for therapy. The present study suggests that the increased levels of p27, and its binding and inhibition of cyclin E/cdk2 contribute to G1 arrest of LNCaP human prostate cancer cells in response to high doses of DHT.

Several other reports suggest a role for p27 as a mediator of G1 arrest in prostate cancer tissue culture models. G1 arrest of prostate cancer line DU145 induced by EGF receptor blockade involves upregulation of p27 protein and corresponding inhibition of cyclin E/cdk2 activity (Peng *et al.*, 1996; Zi *et al.*, 1998). A subline of LNCaP developed by continuous culture in the absence of androgen is growth inhibited by low doses of androgen. In this LNCaP subline, loss of cdk2 activity was also correlated with accumulation of p27 (Kokontis *et al.*, 1998). In an androgen dependent mouse mammary carcinoma cell line, SC-3, G1 arrest upon androgen withdrawal is associated with a similar upregulation of p27 and the binding and inhibition of cdk2 by p27 (Menjo *et al.*, 1998). In these different tissue culture models, androgen mediates different effects on proliferation: in the one case, androgen induces growth arrest, whereas in the other, androgen withdrawal has this same effect. Interestingly, in each case, a similar mechanism of G1 arrest appears to be invoked, with upregulation of p27 leading to inhibition of cyclin E/cdk2 activity. Since the growth of prostate cancers *in vivo* can initially be restricted by anti-androgens, it would be of interest to determine whether p27 is up-regulated following androgen depletion of steroid-dependent prostate cancer.

Increasing data from studies of p27 *in vivo*, in human prostate cancers, suggest a role for this KIP in prostate epithelial cell growth. Several recent reports indicate a prognostic role for p27 in prostate cancer. Low or undetectable levels of p27 protein in primary prostate carcinoma have been shown to correlate with increased proliferative index (Guo *et al.*, 1997), increasing tumor grade (Tsihlias *et al.*, 1998; Yang *et al.*, 1998; Guo *et al.*, 1997; Cote *et al.*, 1998) shorter disease-free survival (Tsihlias *et al.*, 1998;

Yang *et al.*, 1998; Cote *et al.*, 1998; Cordon-Cardo *et al.*, 1998), and decreased overall survival (Cote *et al.*, 1998). In our study of the prognostic value of p27 in human prostate cancer, strong p27 staining was uniformly seen in benign prostatic epithelial components in all tumor sections. p27 staining was variable in prostatic intraepithelial neoplasia and reduced in most prostate cancers. A small subset of tumors, treated with pre-operative androgen ablation therapy prior to radical prostatectomy, tended to show higher expression of p27 protein than that in untreated cases. The few tumors that showed a persistence of low p27 staining (less than 25% of tumor nuclei positive) after androgen ablation therapy had the worst prognosis (Tsihlias *et al.*, 1998). In their study of p27 expression in prostate cancer, Cordon-Cardo *et al.* (1998) found low or absent p27 in androgen-independent metastatic prostate cancers. These authors also raised the possibility that mechanisms leading to accelerated p27 degradation may contribute to the development of metastases and/or progression of prostate cancer to the androgen-independent state. Taken together, both studies of p27 in prostate cancer cell lines and in primary tumors raise the hypothesis that changes in pathways that regulate p27 levels may contribute to tumor progression and to growth modulation of human prostate cancer cells in response to various androgenic stimuli.

Materials and methods

Cell culture

The fast growing strain of LNCaP, LNCaP-FGC (Berns *et al.*, 1986), and cell lines PC-3 and DU145 were purchased from ATCC. Cells were grown in RPMI 1640 culture media plus 5% FBS without phenol red. All experiments were performed using LNCaP passages 25 to 40. Cells were grown to 80% confluence in 10 cm tissue culture plates and split 1:6. Cell populations growing asynchronously were then treated with variable concentrations (0.1–100 nM) of 5 α -dihydrotestosterone (Sigma Laboratories, St. Louis, MO, USA) or ethanol vehicle (0.1% and 1.0%) alone as a control. Cells were treated for up to 96 h, and the media changed every 48 h. As controls, the AR-negative prostate cancer lines PC-3 and DU145 were cultured in DMEM supplemented with 5% FBS without phenol red. Asynchronously growing PC-3 or DU145 cells were treated with 100 nM DHT for 48 h prior to recovery for flow cytometry and protein analysis.

Determination of PSA secretion

Cells were cultured on 10 cm plates in 10 ml media. At 30% confluence, cells were washed twice with PBS and then cultured with fresh medium with or without addition of 100 nM DHT. Conditioned media were collected at 48 h and PSA concentration determined using the Hybritech assay.

Immunohistochemistry

LNCaP cells were plated on poly-L-lysine coated glass slides and cultured for 24 h. The media was then supplemented with 100 nM DHT and cells cultured for a further 48 h. Cells were then washed in PBS, fixed in 4% paraformaldehyde containing 0.2% Triton X-100 for 10 min at room temperature. Slides were then blocked with 3% hydrogen peroxide in methanol followed by normal horse serum (10% solution) and then incubated overnight at 4°C with anti-p27 monoclonal antibody (Transduction Laboratories, Lexington,

KY, USA) diluted 1:1000 (0.25 µg/ml) in PBS. Slides were then reacted with biotin-labeled anti-mouse IgG and incubated with preformed avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA). Metal-enhanced diaminobenzidine substrate (Pierce, Rockford, IL, USA) was then added in the presence of horseradish peroxidase. Cells were photographed using a Wilovert microscope equipped with a Wild Leitz MPS 52 photocamera (Wild Leitz Ltd., Heerbrugg, Switzerland).

Flow cytometric analysis

Cells were pulse-labeled with 10 µM bromodeoxyuridine (BrdU) for 2 h at intervals after addition of DHT to asynchronously growing cells. Cells were then harvested, fixed with 70% ethanol, treated with 0.1 N HCl, and heated for 10 min at 95°C to expose labeled DNA. Cells were then stained with anti-BrdU-conjugated FITC (Becton Dickinson) and counterstained with propidium iodide. Cell cycle analysis was carried out on a Becton Dickinson FACScan, using Cell Quest software.

Immunoblotting

Cells were lysed in ice cold NP-40 lysis buffer (0.1% NP-40, 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonylfluoride, and 0.02 mg/ml each of aprotinin, leupepsin, and pepstatin). Lysates were sonicated and clarified by centrifugation. Protein was quantitated by Bradford analysis and 20–100 µg protein per lane resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Transfer and blotting was as described (Dulic *et al.*, 1992). Proteins were detected by electrochemiluminescence (ECL). Densitometry was performed using the Molecular Dynamics Imaging system and ImageQuant software to quantitate the relative amounts of p27 protein detected on Western blots. For detection of cyclin E-associated proteins by immunoprecipitation-Western analysis (IP-Western), cyclin E was immunoprecipitated from 200 µg protein lysate, complexes resolved, blotted and blots reacted with cyclin E, cdk2, p21, and p27 antibodies. For detection of cdk4 complexes, 100–400 µg protein lysate was immunoprecipitated with cdk4 antibody and associated cyclin D1, p15, p16, p21, and p27 proteins detected by immunoblotting. For immunodepletion of p27, p27 was serially immunoprecipitated three times from 200 µg protein lysate and then cyclin E was immunoprecipitated from the p27-depleted lysate. The amounts of immunoprecipitable cyclin E protein, associated cdk2 and p27 proteins, and kinase activities prior to and after p27 immunodepletion were compared using IP-Western blotting and IP-kinase assays.

Oligonucleotide transfections

The sequences of the GS5422 antisense (ASp27) and mismatch p27 GS5585 (MSP27) C-5-propyne modified phosphorothioates used were 5'-TGGCTCTCXTGCGCC-3' and 5'-TGGCTCXCTTGCGCC-3', respectively. X indicates the proprietary 'G-clamp' modification of these oligonucleotides, provided by Gilead Sciences. Asynchronously growing cell cultures were transfected with oligonucleotides at a concentration of 50 nM using cytofectin G3815 at 2.5 µg/ml, as described (St.Croix *et al.*, 1996). After 6 h the transfection

cocktail was removed and replaced with complete medium containing 100 nM DHT. Cells were recovered for flow cytometric and protein analysis 36 h thereafter. 100 nM DHT-arrested cells were also transfected with ASp27, however, the reduction of p27 was considerably less in these cells than after transfection of ASp27 into asynchronously growing cells. The minor reduction in p27 protein levels in these DHT arrested cells was not sufficient to cause release from G0.

Cyclin-dependent kinase assays

Cdk4 kinase assays were performed using the method of Matsushima *et al.* (1994), using a truncated recombinant retinoblastoma protein as substrate. Quantitation of radioactivity incorporated in the substrate was performed using a Molecular Dynamics PhosphorImager and ImageQuant software. Cyclin E-associated kinase assays were performed using either a monoclonal anti-cyclin E antibody (mAb E172, from E Harlow, Mass. General, MA, USA) or a polyclonal anti-cyclin E antibody (from D Agarwal, Lee Moffat Cancer Center, FL, USA). Histone H1 was used as substrate for cyclin E-associated kinase assays. In each case background activity was determined using a non-specific antibody as a control. Background activity was subtracted, and kinase activities were graphed as a per cent maximum activity.

Antibodies

The following antibodies were used in the immunoblotting experiments: pRB mouse mAb from Pharmingen; cdk2 rabbit pAb sc-163, cdk4 rabbit pAb sc-260, cdk6 rabbit pAb sc-172, cyclin D1 mouse mAb HD11, cyclin A rabbit pAb sc-596, cyclin B1 mouse mAb sc-245, and p21 rabbit pAb sc-397, all from Santa Cruz Biotechnology, CA, USA; p27 mouse monoclonal antibody from Transduction Laboratories, Lexington, KY, USA. PSA antibody was purchased from DAKO, Denmark. Monoclonal PSTAIRE antibody was a gift from S Reed (The Scripps Research Institute, CA, USA); cyclin D1 mouse mAb DCS-11 and p16 mouse mAb DCS-50, from J Bartek (Danish Cancer Society, Denmark); E12 and E172, mouse monoclonal antibodies to cyclin E from E Harlow (Mass. General, MA, USA); and cdk4 rabbit polyclonal antibody, for use in immunoprecipitation of cdk4, was provided by D Beach (CSH Labs, NY, USA). A monoclonal antibody, JC-6, which recognizes the third ankyrin repeat of human p16 (Enders *et al.*, 1995) and cross reacts with human p15, was used for immunoblotting of p15 in these studies. Results were confirmed by repeat biologic assays with different cell lysates.

Acknowledgments

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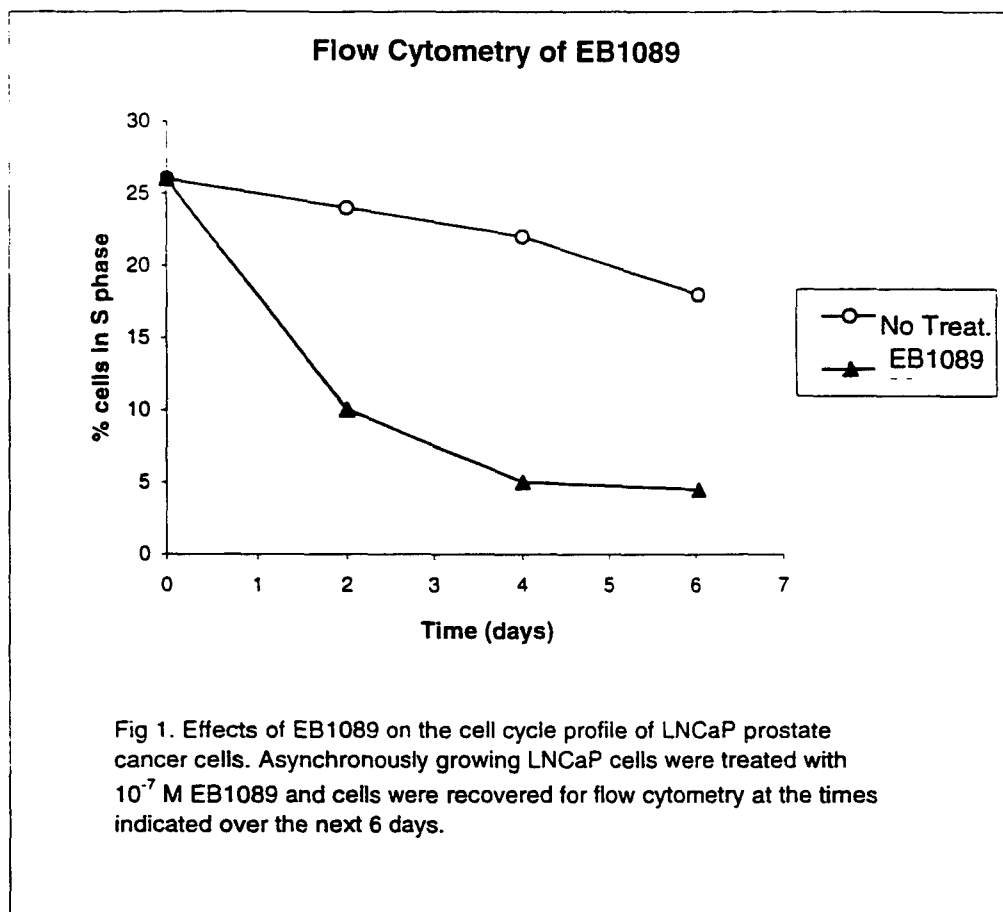
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Figure 1



EB1089 effects on cell cycle regulators

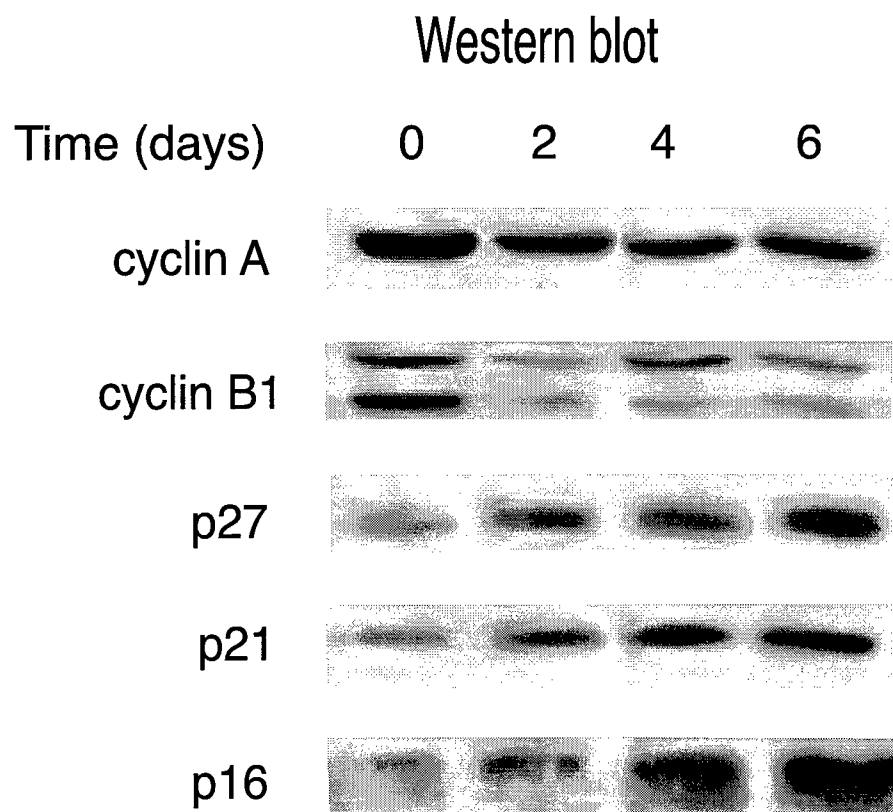
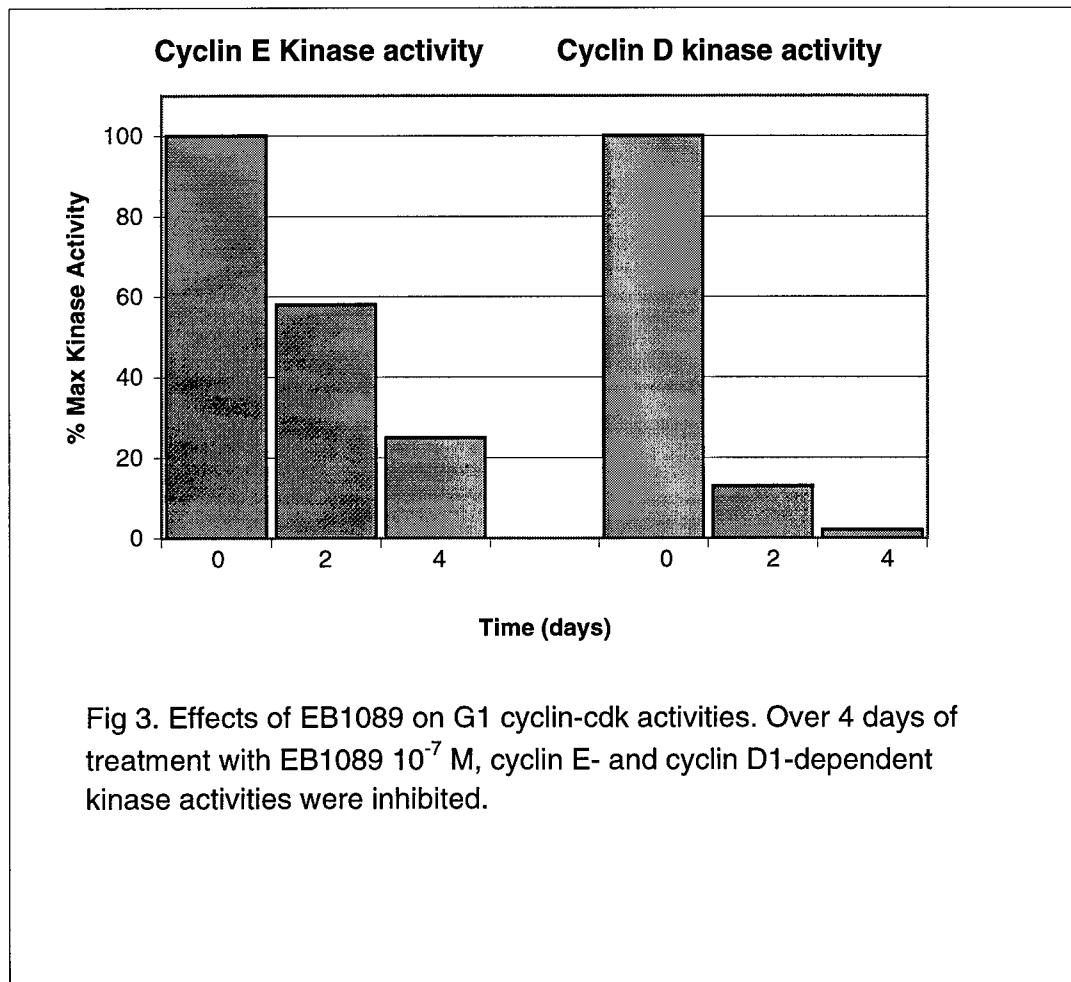


Fig 2. Effect of EB1089 on cell cycle regulators. As cells entered G1 arrest over 6 days of drug treatment, levels of cyclin A and cyclin B1 fell and the cdk inhibitors p21, p27 and p16 increased. Levels of cyclin D1, cyclin D2, cyclin E and cdk2, cdk4 and cdk6 did not change (not shown).



EB1089 10-7 M

IP p21

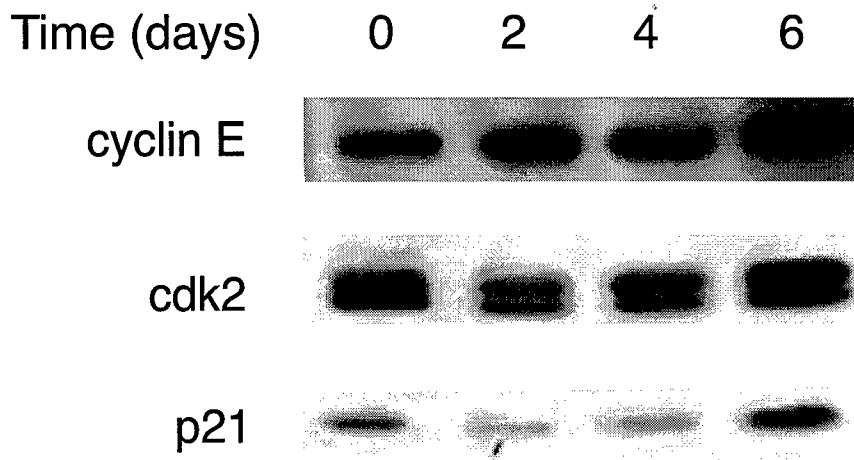


Fig 4. EB1089 causes an increase in p21 and an increase in p21 binding to cyclin E-ckd2. p21 was immunoprecipitated from cell lysates before (T=0) and 6 days after EB1089 treatment. Complexes were resolved on SDS-PAGE, blotted and p21-associated proteins detected by immunoblotting.

EB1089 10-7 M

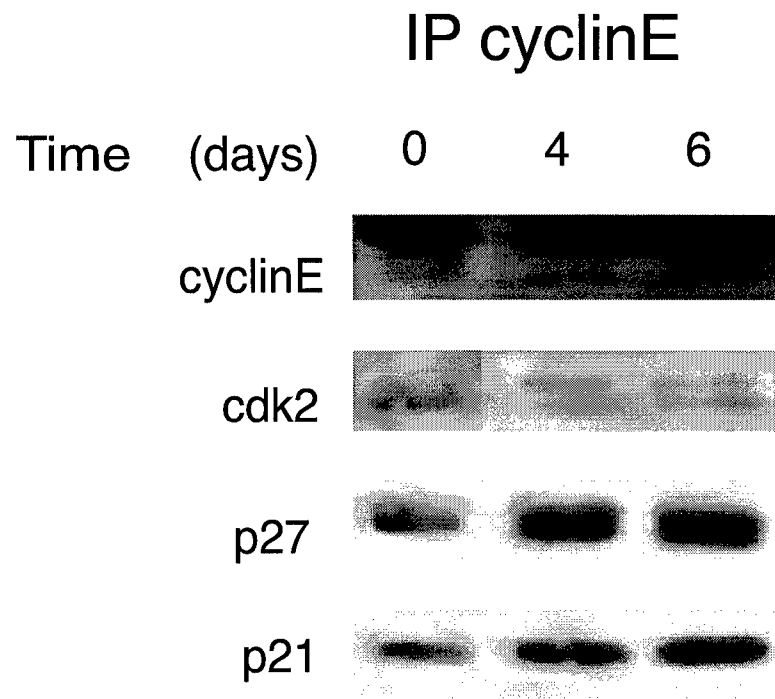


Fig 5. Binding of cdk inhibitors p21 and p27 to cyclin E-cdk2 is increased by EB1089. Over 6 days of EB1089 treatment, levels of cyclin E-bound p21 and p27 increased significantly. Cyclin E levels were not changed by EB 1089 treatment.

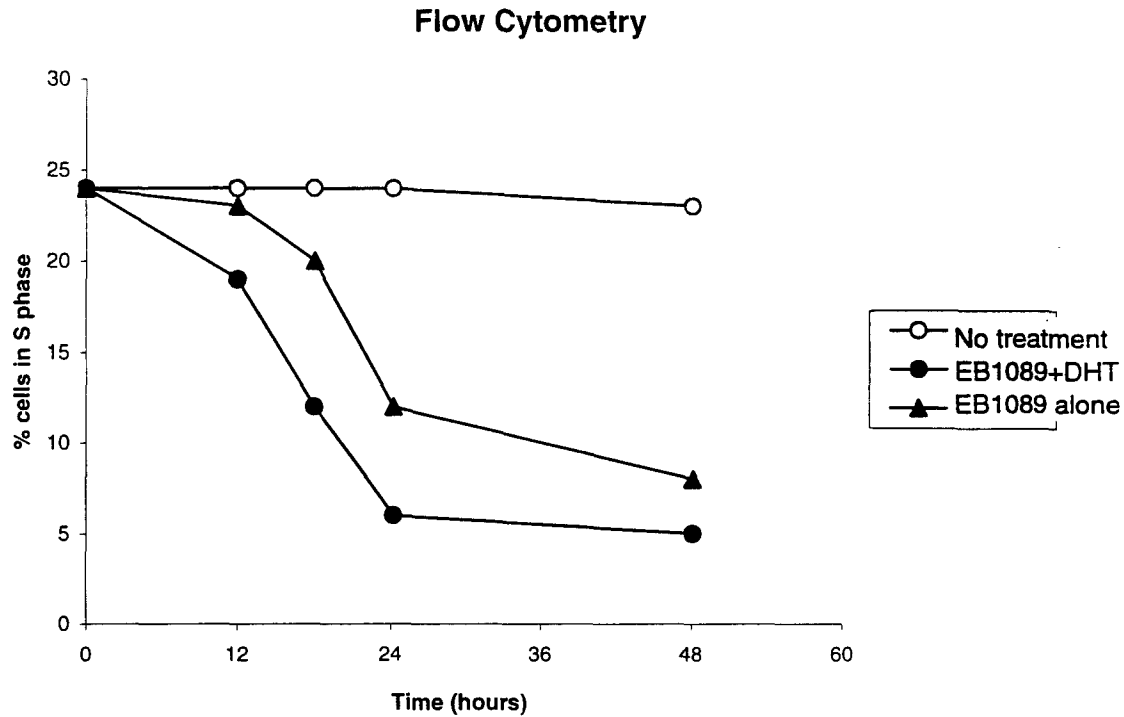


Fig 6. Asynchronous cultures of LNCaP were treated with EB1089 10^{-7} M alone, EB1089 10^{-7} M+DHT 0.3 nM, DHT 0.3 nM alone (not shown), or no drug. Cells were recovered at the indicated times for flow cytometric analysis. The % cells in S phase are graphed with time. DHT alone caused essentially no change in the cell cycle. EB1089 with DHT caused more rapid and profound inhibition of cell cycle progression than did EB1089 alone.

EB1089 10⁻⁷ M+DHT 0.3nM

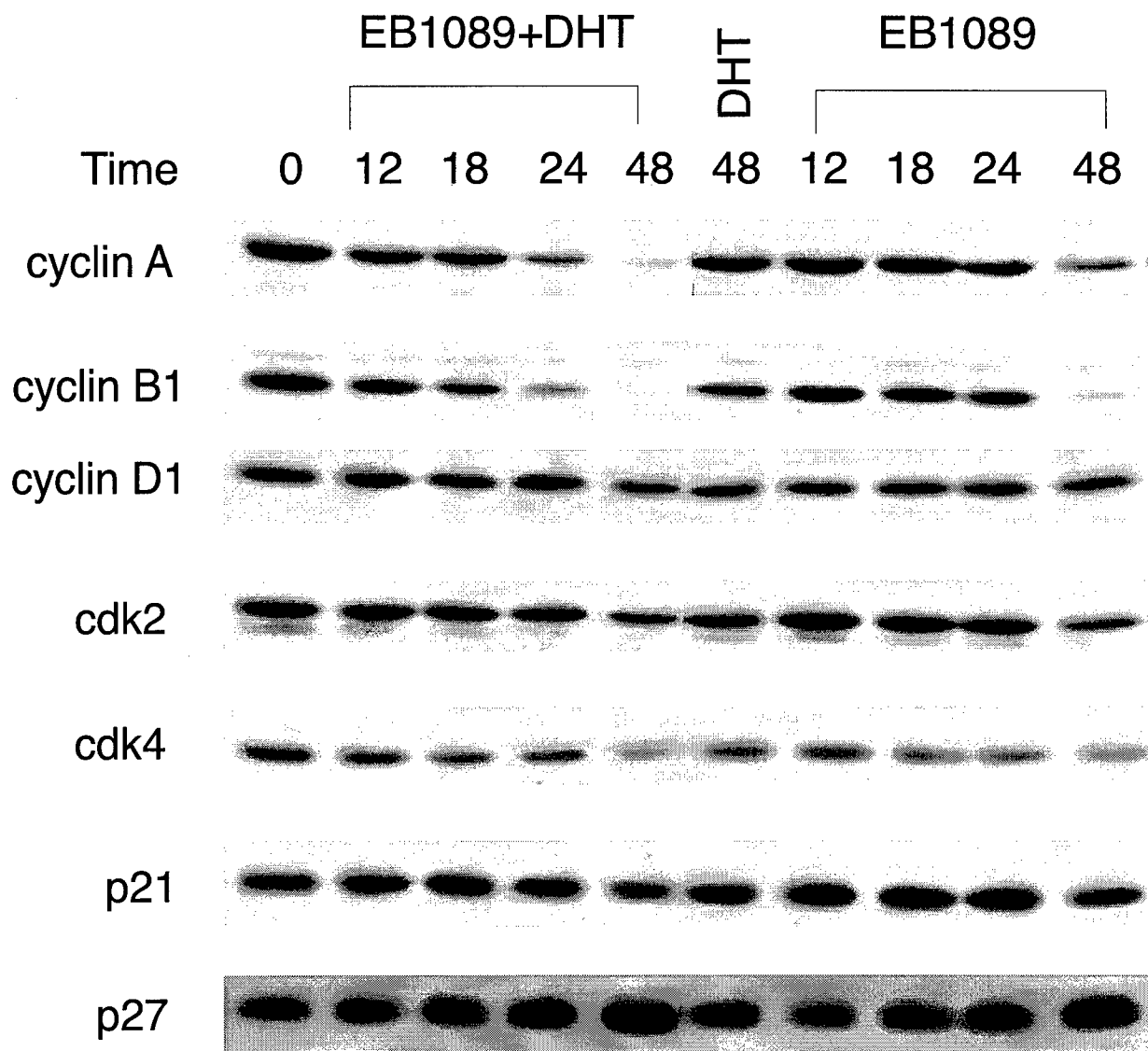


Fig 7. Comparison of effects of EB1089 alone and EB1089 with DHT on cell cycle regulators. The combination of EB1089 10⁻⁷ M with 0.3 nM DHT caused a more rapid loss of cyclins A and B1, and a more rapid rise of p27 than did EB1089 alone. Levels of cdk2, cdk4, cyclin D1 and cyclin E were not affected significantly by either drug treatment. There was little change in p21 within 48 hours. While p21 levels rose with EB1089, the increase was most notable at 4 to 6 days (not shown).

EB1089 10⁻⁷ M+DHT 0.3nM

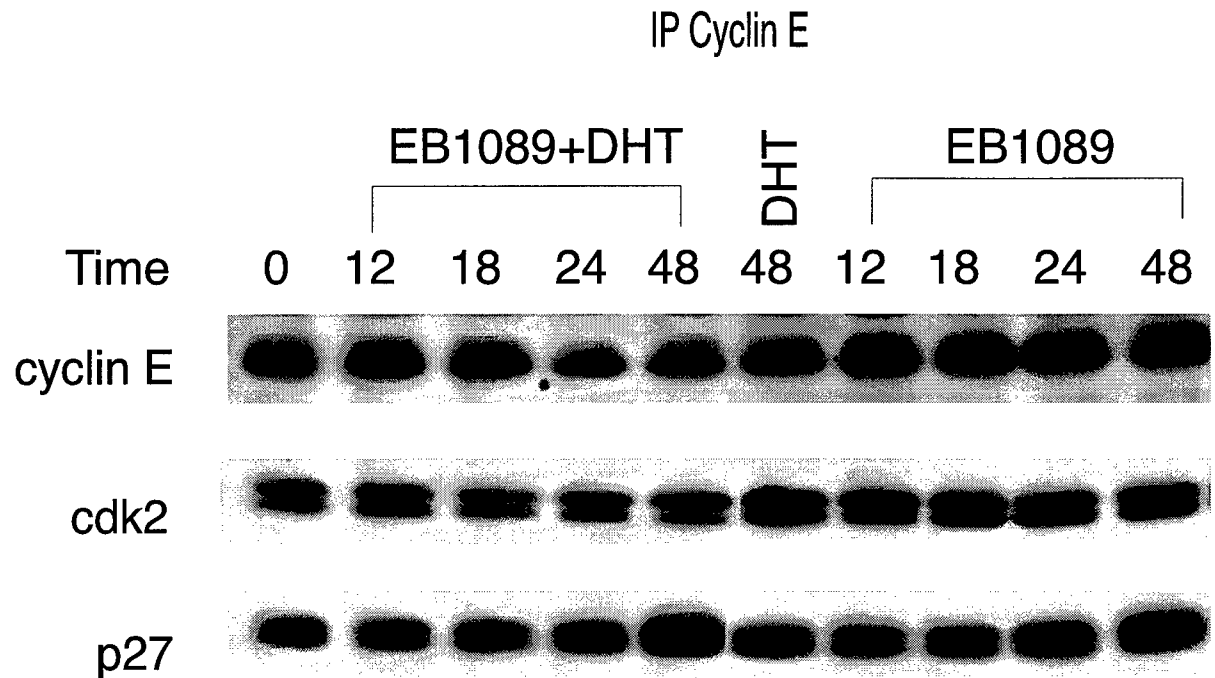


Fig 8. EB1089 and DHT cause a greater increase in p27 binding to cyclin E-cdk2 than EB1089 alone. At intervals following addition of the indicated drugs, cells were lysed and cyclin E immunoprecipitated. Cyclin E immune complexes were resolved and associated proteins were detected with the indicated antibodies. There was a 5 fold increase in p27 binding to cyclin E in the EB1089+DHT treated cells. p21 did not increase in cyclin E complexes during the first 48 hours in any treatment group.

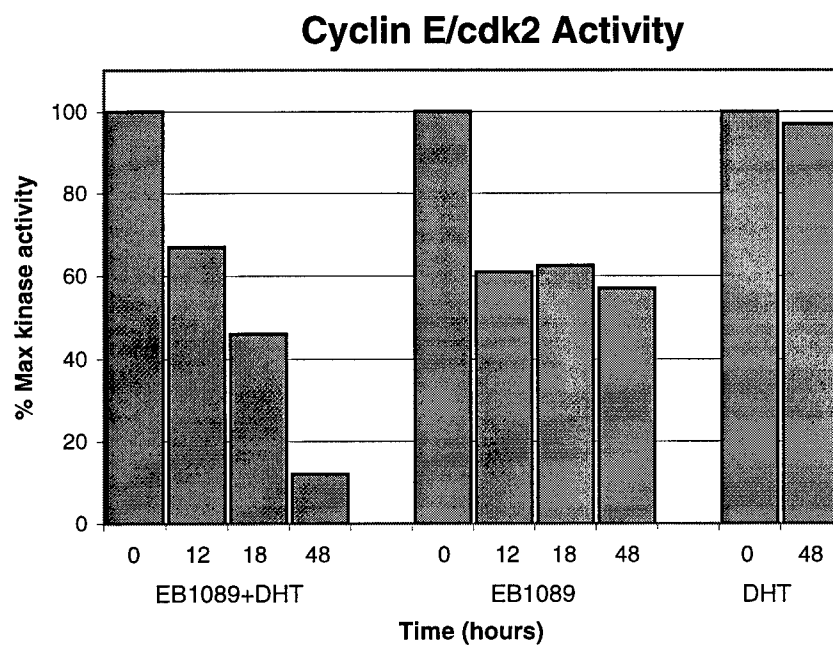


Fig 9. Drug effects on cyclin E-cdk2 activities. The inhibition of cyclin E-cdk2 by EB1089+DHT was more rapid and more profound than that caused by EB1089 alone. There was essentially no inhibition of cyclin E-cdk2 activity by DHT 0.3 M alone.



Animal Use Protocol Modification Application

For Office Use Only

Expiry
Date

14 Jun 00

Protocol# 99-029

Complete sections 1 & 2 in FULL. For all other sections, document only the information which has CHANGED from the approved protocol.

1 Principal Investigator	Surname	First Name	Initial
	Slingerland	Joyce	M
	Department/Faculty		
	Cancer Biology Research		
	Business Telephone	Laboratory Telephone	Residence Telephone
	(416) 480-6100 Ext.3494	(416) 480-6100 Ext.3495	(416) 924 2725
	Pager	E-mail Address	
	(416) 232 7077	joyce.slingerland@utoronto.ca	

2 Project	Project Title
	In vivo assays of growth suppression of prostate cancer xenografts by vitamin D3 analogs and dihydrotestosterone.

3 Funding Changes	Internal/External	Peer Reviewed?	Name of Agency/Source

4 Emergency Contact Changes	A.Faculty - Name (mandatory)	Rank	Emergency Telephone
	B.Other (optional)	Rank	Emergency Telephone

5 Research Staff Changes	Name	Add/Remove	Position	Training
				<input type="checkbox"/>
				<input type="checkbox"/>

6 Experimental Design Changes	Describe modification to experimental design: Is this a change to an existing procedure? <input type="radio"/> or an addition? <input checked="" type="radio"/>	
	Terminal blood collection will be done in some mice by cardio centesis using a 1 ml syringe and a #23 gauge needle under anaesthetic.	
Outline reason(s) for the modification:		

We require a minimum sample size of 0.5 ml in order to have the blood tested.

7 Change in Animals	Animal Strain	# of Animals	Sex	Age/Weight	Source of Animals	Location of Holding	Location of Experiment

8 Signatures	Principal Investigator or Course Director	Signature	Date
	Chairperson Local Animal Care Committee	Signature	Oct 28/99
	Veterinarian	Signature	Jan 11/00
		Signature	10/01/2000

Animal Use Protocol

IMPORTANT! Incomplete protocols will be returned!

For Office Use Only

New Protocol# 99-029

Expiry Date 14 Jun 00

☒ New Project

☐ 4th yr. Renewal

Previous Protocol#

1 Principal Investigator or Course Director

Surname **Slingerland** First Name **Joyce** Initial **M** Personnel No.

Department/Faculty **Cancer Biology Research** Position/Rank **Senior Scientist**

Business Telephone (416) 480-6100 Ext. 3494 Laboratory Telephone (416) 480-6100 Ext. 3495 Residence Telephone (416) 924 2725

Pager (416) 232 7077 E-mail Address **joyce.slingerland@utoronto.ca**

Mailing Address **Sunnybrook & Women's College Health Sciences Centre, S218, 2075 Bayview Ave., Toronto, ON M4N 3M5, Canada**

NAME OF DESIGNATED ALTERNATE FOR EMERGENCIES

A.Faculty - Name (mandatory) **Jorge Filmus** Rank **Senior Scientist** Emergency Telephone **(416) 406 0627**

B.Other (optional) Rank Emergency Telephone

2 Project

Project Title

In vivo assays of growth suppression of prostate cancer xenografts by vitamin D3 analogs and dihydrotestosterone.

3 Funding

☒ External Agency

☐ Internal Source

Grant Account # 724179322

Status: ☒ Awarded ☐ Pending ☐ Peer Reviewed (by whom?)
☒ Peer Reviewed ☐ Non-Peer Reviewed ☐ Non-Peer Reviewed

4

PROPOSED START DATE Day 15 Month June Year 1999 EXPECTED COMPLETION DATE Day 15 Month June Year 2000

5

CATEGORY OF INVASIVENESS

☐ A ☐ B ☐ C ☒ D ☐ E

6

TYPE OF EXPERIMENT (Check all applicable boxes)

☒ Research ☐ Teaching ☐ Testing ☐ Research/Teaching

SURGICAL ☐ Acute ☒ Survival

NON-SURGICAL ☐ Acute ☐ Chronic

All animals in this protocol will be maintained and used in accordance with the current recommendations of the Canadian Council on Animal Care, the requirements under the Animals for Research Act, R.S.O. 1980, and Sunnybrook Health Science Centre Animal Care Committee Policies and Guidelines.

Principal Investigator or Course Director

Joyce M. Slingerland

Signature

Date

June 3, 1999

Chairperson Local Animal Care Committee

Signature

Date

Sept 29 / 99

Veterinarian

Signature

Date

29/09/99

Approvals Declaration

In LAYMAN'S TERMS, BRIEFLY describe all procedures and techniques to be used; giving a DETAILED description of ONLY those performed on animals. Space for this description is limited; attach 1 ADDITIONAL PAGE if necessary.

I. RATIONALE

One of the major forms of therapy for advanced, metastatic prostate cancer is androgen ablation. This therapy aims to reduce the available amount of male hormone or androgen that can stimulate growth of prostate cancer cells. This is accomplished either by surgical removal of the testes (orchidectomy) or by a medical orchidectomy using various forms of medication that block the effects of androgen. Unfortunately, blockade of androgen effects in men has significant negative side effects.

Over the last year and a half we have been studying the growth inhibitory effects of high dose androgen or dihydrotestosterone, and of vitamin D3 analogs including an analog EB1089, produced by the Leo Co. in Denmark. Both of these agents, used on their own, cause growth arrest in tissue culture of the prostate cancer cell line LNCaP. However, the dose of androgen or DHT that causes growth arrest in prostate cancer in tissue culture is much higher than the normal level in the human male. On their own, low doses of dihydrotestosterone do not inhibit prostate cancer growth. Recently, we have found that when a low, physiologic dose of dihydrotestosterone is added to the usual dose of vitamin D3 analog EB1089, the two drugs act synergistically to cause a more rapid and more complete arrest of prostate cancer cells growth. We wish now to explore the possibility that a combination of low, physiologic doses of dihydrotestosterone with EB1089 may mediate arrest of prostate cancer cells in an experimental model of human prostate cancer, that is human prostate cancer cells grown in an immunodeficient or nude mice. We plan to ascertain whether this low, physiologic dose of dihydrotestosterone, administered through an indwelling subcutaneous pellet to the mouse and oral administration of EB1089 can either prevent the development of prostate cancer tumors when these are injected subcutaneously in the mouse, or can inhibit the growth of tumors once they are established. Studies of this nature in the mouse are essential to provide the data that would allow the next step, which is to proceed with clinical trials of these novel forms of relatively innocuous hormones in human prostate cancer patients. This vitamin D3 analogs have been specifically designed to inhibit prostate cancer growth and yet they do not cause the negative side effects of hypercalcemia, that is, the increase in serum calcium that is normally caused by vitamin D3 itself. Low dose dihydrotestosterone is currently in use clinically to prevent osteoporosis, the loss of muscle mass and the loss of libido that accompany human male aging. Low dose androgen is currently not used for prostate cancer except in certain clinical trials, because of its potential to promote prostate cancer growth. We wish to explore the possibility that, in combination with vitamin D3 analogs such as EB1089, low dose androgen may have its positive side effects on the human subject and rather than promoting prostate cancer growth, may indeed have a synergistic effect to inhibit prostate cancer growth. We have documented extensively in tissue culture this growth inhibitory effect on prostate cancer over the last year and a half. Currently EB1089 is being used in clinical trials for prostate cancer in humans. The LNCaP and Shionogi cancer cell lines have been chosen for use in these experiments because they are sensitive to androgen and vitamin D3, and also because they form subcutaneous nodules when injected into the mice strains indicated below. They do not metastasize. Although these tumors will grow as subcutaneous nodules, the fact that they do not have a tendency to spread to other organs in the mouse will minimize animal suffering. The subcutaneous tumors that arise do not cause the animals to be systemically sick.

II METHODS

A Cell Lines. Human prostate cancer LNCaP cells, passage 40, are grown in RPMI 1640 with 5% fetal bovine serum (FBS). The Toronto subline of the transplantable SC-115 Shionogi mouse mammary carcinoma will be used.

B In Vivo Tumour Growth. 6- to 8- week old male athymic nude mice (CrI:nu/nu(CD-1) BR) will be inoculated sc with 1×10^6 LNCaP cells suspended in 100 μ L Matrigel. 2×10^7 Shionogi cells will be injected into male DD/S mice. Tumours are measured twice weekly and their volumes calculated by the formula $L \times W \times H \times 0.5236$ (7). Blood samples for sequential DHT and PSA measurements will be obtained as previously described. Blood will be taken from the tail vein of the mice for serum calcium and for PSA and testosterone levels. Samples will not exceed 0.2 ml and there will be a waiting time of two weeks between sample takings.

C Surgical Castration. Inject 0.20-0.25 ml of anesthesia IP. Wipe belly and lower abdomen with iodine swab. When mouse does not respond to pinching or squeezing his foot, make incision midline through skin. Cut through muscle wall being careful not to cut colon. Using forceps, pull left testicle and epididymal fat pad

out of body. Tie three knots below testicle and fat pad with 3.0 chromic gut thread. Cut off testicle and fat pad above knots. Repeat for right testicle and epididymal fat pad. Stitch muscle wall by pulling a needle and 3.0 chromic gut thread through muscle on both sides of the incision and making three knots. Repeat at another point along the incision. Be careful not to pierce internal organs. Clamp skin together with 1 or 2 staples. The staples should be removed after 10-14 days. The post-doctoral fellow who will do these experiments is a urologic surgeon.

D Histology and Immunohistochemistry. Tumors will be excised from sacrificed mice and processed for histological analysis as previously described. Deparaffinized and frozen sections will be incubated with monoclonal antibodies prepared against PSA, p27, and MIB1 (Ki-67), and digoxigenin-linked nucleotide and N-terminal deoxynucleotidyl transferase (Apotag, Oncor, Inc.) Frozen section will also be used for in situ hybridization to analyze changes in mRNA levels and distribution of these markers when appropriate.

E Western Analysis. Tumour specimens harvested at various times pre- and post-castration and following treatment with DHT and EB1089 will be processed for cytoplasmic and nuclear protein extraction and probed for antibodies against AR, p27, VDR, and actin as a loading control.

F RNA Isolation and Northern Blot Analysis. Gene expression will be tested by Northern analyses using standard techniques.

G Cell cycle profiles. The distribution of tumor cells in the different cell cycle phases will be monitored using tumor dissociation according to the Hedley method and routine preparation for FACS analysis.

II. EXPERIMENTAL DESIGN.

A. Determination of physiologic DHT doses and normocalcemic doses of EB1089 in mice. Several mice will be used to assay the serum levels of DHT following implantation of subcutaneous DHT pellets in castrate mice. We will use 12.5 mg subcutaneous pellets which should provide serum levels of 3-5 ng/ml in the mouse. Our goal is to approximately the serum levels of DHT in non-castrate mice without DHT pellets (eg 3-5 ng /ml serum). While EB1089 does not cause hypercalcemia in humans, it will be necessary to demonstrate that the doses recommended by the manufacturer, Leo, for use in mice (0.1-1.0 µg/kg/day) do not cause any negative side effects, including hypercalcemia in the mice. Both assays of DHT and calcium can be performed in the hospital's biochemistry department.

B. Effects of DHT and EB1089 to suppress tumor formation

We will test whether low dose DHT (delivered by sub-cutaneous pellet) and EB1089 can prevent tumor formation as follows. Mice will be injected at a maximum of 4 sites (max. vol. of 200 µl/site) sc with the respective tumor lines after a 2 week pre-treatment with DHT, DHT +EB1089, EB1089 alone or no treatment (10 mice each treatment category). Hormonal treatments will continue for the duration of the experiment. Tumor number and size and markers of proliferation and DNA analysis will be monitored at the termination of the experiment. The termination of the experiment will be defined as follows: Animals will be sacrificed 6 months after tumors arise or when the tumor reaches a mean subcutaneous flank tumor diameter of 17 mm, that is a volume exceeding (17 mm)³. Any animal bearing an ulcerated tumor will be sacrificed as soon as ulceration is detected.

C. Effects of DHT and EB1089 to suppress established tumor growth

DHT supplemented castrate mice will be injected at 4 sc sites per animal as above with either LNCaP or Shionogi cells and tumors allowed to establish over a 4 wk. Period. We anticipate tumor sizes of <0.5 cm after 4 wks. After 4 weeks, hormonal treatment will begin. 10 Animals will be placed in each of 4 experimental arms: 1) no treatment controls 2) DHT 3) EB1089 4) both DHT and EB1089. Tumor size and parameters of proliferation and p27 levels will be monitored at 1, 2, 3, and 4 months or until the tumor reaches a mean subcutaneous flank tumor diameter of 17 mm whichever occurs first. Two mice will be sacrificed and tumors will be harvested prior to and 1, 2, 3 and 4 months after their appearance and divided into 3 portions: an unfixed portion will be used for FACS analysis; one portion will be fixed in 10% NBF and embedded in paraffin for morphological analysis; a third portion will be frozen at -80°C for Western blots and for RNA extraction and northern analyses. H&E and Apotag staining will be used to evaluate castration-induced cell death with characteristic apoptotic cells. Immunostaining using antibodies directed against the markers mentioned above will be performed to evaluate qualitative and semi-quantitative (distribution and intensity) changes in respective protein levels following hormonal therapies (DHT and or EB1089). Internal reference slides will be used to normalize for variation in intensity of immunostaining between preps.

8

PRIMARY OBJECTIVE(S) OF THIS PROJECT

Summarize the primary objective(s) and benefits expected from the study in terms suitable for NON-scientific readers.

Objectives

We have found that a combination of low, physiologic doses of dihydroxytestosterone and vitamin D3 analogs cause a synergistic growth inhibitory response in prostate cancer cells in culture. We wish to test whether low dose dihydrotestosterone and vitamin D3 analogs given concurrently to mice can prevent the growth/development of prostate cancer xenografts or can cause xenograft tumors, once established, to regress.

9

NOTE: University of Toronto 1-day course is MANDATORY for new graduate students, research technicians and technologists, research assistants/associates, and post-doctoral fellows.

*A box with an "x" indicates that the staff member has completed or is registered for the U of T Animal Care Course, or equivalent training.

Name	Department	Position	Training*
William Zhang	Cancer Biology Research	Post-Doctoral	<input checked="" type="checkbox"/>
Joyce Slingerland	Cancer Biology Research	Senior Scientist	<input checked="" type="checkbox"/>
			<input type="checkbox"/>
			<input type="checkbox"/>
			<input type="checkbox"/>
			<input type="checkbox"/>
			<input type="checkbox"/>

10

Animals to be Used

Animal Species (Common Name)	Strain	# of Animals	Sex	Age/Weight	Source of Animals	Location of Holding	Location of Experiment
Mice	CB17SCID	130	M	4-8 wks	Charles Rivers	SB	SB
	DD/S	130	M	6-8 wks	Charles Rivers	SB	SB

ANIMAL HOUSING REQUIREMENTS ☐ Standard Cages ☒ Microisolator Cages ☐ Other

11

Justification

JUSTIFICATION FOR: A. Species and B. Number of Animals Used Please limit justification to the space provided.

A. CB17 SCID

mice will be used for xenografting the human prostate cancer line LNCaP. Syngeneic DD/S strain mice will be used for the in vivo tumor studies with the murine Shionogi hormone sensitive cancer line.

- B. We propose to use 10 animals from each strain (CB17 SCID) and DD/S) to assay baseline effects of castration and use of DHT s.c. pellets on DHT levels. Vitamin D3 analogs may cause hypercalcemia. We will ensure that doses of EB1089 administered po (0.1-1.0 mg/day) do not cause hypercalcemia in the experimental animals. Physiologically equivalent doses do not cause hypercalcemia in humans. The 2 experiments detailed in Part 7 A involves 10 animals in each of the 4 arms for each of the LNCaP and Shionogi cell line experiments. Part 7B involves 20 mice in each of the 4 arms for each of the 2 cancer cell lines. We estimate that this number is probably the minimum required to allow meaningful interpretation of results.

12

ALTERNATIVES

Are non-animal alternatives available for this project?

☐ Yes☒ No

If Yes, explain why they have been rejected.

Alternatives

This project is specifically designed to test, in an animal model, growth inhibitory effects of EB1089 and DHT on prostate cancer that we have studied extensively already in tissue culture. The next step in moving these relatively innocuous hormonal treatments into clinical trials for human prostate cancer, is to test these drugs on prostate cancers in an animal model.

13

Drugs Used

DRUGS USED FOR ANAESTHESIA/ANALGESIA

DRUG

DOSAGE

ROUTE OF ADMINISTRATION

A. Pre-Anaesthesia

Pre-Anaesthesia

IP

B. Anaesthetic Induction:

ketamine , xylazine

IP

Anaesthetic Maintenance:

C. Analgesic(s)

D. Other

Other

E. None ☐

14

Euthanasia

☒ Anaesthetic Overdose (specify agent)☐ Carbon Dioxide☒ Cervical Dislocation (small rodents only)☐ Decapitation☐ Exsanguination (under anaesthesia)☐ Other (specify)

15

Hazardous Agents

☐ Biological (specify)

Biohazard Safety Committee Approval

☐ Chemical

Occupational Health & Safety Dept. Approval

☐ Carcinogen

Occupational Health & Safety Dept. Approval

☐ Radioisotope/Radiation

R/A Permit #

Expiry Date

☒ None

ANIMAL CARE COMMITTEE USE ONLY - ADDITIONAL NOTES

Animal Use Data Form

Completion of this form is a requirement of the Canadian Council on Animal Care (CCAC).

For a protocol to be considered by the SHSC Animal Care Committee, this form must be completed and submitted with the protocol form.

Institution Name: Sunnybrook Health Science Centre

Institution Code: MH02

Protocol#	Category ¹	Protocol Description ²	PAU ³	Species ⁴	AR ⁵	AU/Yr
99-029	D	An investigation of whether prostate cancer tumors grown subcutaneously in castrated mice can be prevented or growth inhibited by vitamin D3 analogs and dihydrotestosterone.	2	Mice	260	
						For Office Use Only

1. Category:	Category of Invasiveness
2. Protocol Description:	Please give a descriptive protocol title that indicates, in LAY TERMS, the nature of the procedures used (preferably in 40 words or less)
3. PAU	<p>Purpose of Animal Use:</p> <ol style="list-style-type: none"> 1. Studies on a fundamental nature in sciences relating to essential structure or function 2. Studies for medical purposes, including veterinary medicine, that relate to human or animal disease or disorders 3. Studies for regulatory testing of products, for the protection of humans, animals, or the environment 4. Studies for the development of products or appliances for human or veterinary medicine 5. Education and training of individuals in post-secondary institutions or facilities
4. AR	Number of Animals Requested
5. AU/Yr	<p>Number of Animals Used / Year</p> <p>To be completed at the end of each calendar year</p> <p>(If a group of animals is reused in a second protocol, please indicate an "R" and the 1st protocol number next to the number of animals used for the 2nd protocol).</p>

RESEARCH INVOLVING ANIMALS

1. Literature Searches

We have obtained the required information from web sites and literature searches regarding alternatives to painful procedures. Efforts will be made to minimize suffering of experimental animals. Web sites include the following: 1) <http://www.pdk.dgk.ruu.nl/nca>, 2) <http://altweb.jhsph.edu>, 3) The Journal, JAAWS (Journal of Applied Animal Welfare Science).

The experiments proposed must be done in experimental animals. The purpose of this work is to test whether 2 hormonal agents, low dose DHT and EB1089 which we have shown to inhibit prostate cancer cell growth in tissue culture models, are also therapeutically effective in suppression of prostate cancer growth in vivo, in mice.

The results from these experiments will be necessary to achieve proof of principle to allow us to subsequently begin clinical trials of these relatively innocuous hormonal treatments in men with prostate cancers. Evidence of efficacy in an animal model is necessary before human trials can be undertaken.

2. Rationale/Justification for Using Animal

One of the major forms of therapy for advanced, metastatic prostate cancer is androgen ablation. This therapy aims to reduce the available amount of male hormone or androgen that can stimulate growth of prostate cancer cells. This is accomplished either by surgical removal of the testes (orchidectomy) or by a medical orchidectomy using various forms of medication that block the effects of androgen. Unfortunately, blockade of androgen in men has significant negative side effects.

Over the last year and a half we have been studying the growth inhibitory effects of high dose androgen or dihydrotestosterone, and of vitamin D3 analogs including an analog EB1089, produced by the Leo Co. in Denmark. Both of these agents, used on their own, cause growth arrest in tissue culture of the prostate cancer cell line LNCaP. However, the dose of androgen or DHT that causes growth arrest in prostate cancer in tissue culture is much higher than the normal level in the human male. On their own, low doses of dihydrotestosterone do not inhibit prostate cancer growth. Recently, we have found that when a low, physiologic dose of dihydrotestosterone is added to the usual dose of vitamin D3 analog EB1089, the two drugs act synergistically to cause a more rapid and more complete arrest of prostate cancer cell growth. We wish now to explore the possibility that a combination of low, physiologic doses of dihydrotestosterone with EB1089 may mediate arrest of prostate cancer cells in an experimental model of human prostate cancer, that is human prostate cancer cells grown in an immunodeficient or nude mice. We plan to ascertain whether low, physiologic doses of dihydrotestosterone, administered through an indwelling subcutaneous pellet to the mouse and subcutaneous administration of EB1089 can either prevent the development of prostate cancer tumors when prostate cancer cells are injected subcutaneously in the mouse, or whether these drugs can inhibit the growth of tumors once they are established. Studies of this nature in the mouse are essential to provide the data that would allow the next step, which is to proceed with clinical trials of these novel forms of relatively innocuous hormones in human prostate cancer

patients. These vitamin D3 analogs can inhibit prostate cancer growth and yet they do not cause the negative side effects of hypercalcemia, that is, the increase in serum calcium that is normally caused by vitamin D3 itself. Low dose dihydrotestosterone is currently in use clinically to prevent osteoporosis, the loss of muscle mass and the loss of libido that accompany human male aging. Low dose androgen is currently not used for prostate cancer except in certain clinical trials, because of its potential to promote prostate cancer growth. We wish to explore the possibility that, in combination with vitamin D3 analogs such as EB1089, low dose androgen may have its positive side effects on the human subject and rather than promoting prostate cancer growth, may indeed have a synergistic effect to inhibit prostate cancer growth. During the first year of this grant period, we have documented extensively in tissue culture this growth inhibitory effect on prostate cancer. Currently EB1089 is being used in clinical trials for prostate cancer in humans. The LNCaP and Shionogi cancer cell lines have been chosen for use in these experiments because they are sensitive to androgen and vitamin D3, and also because they form subcutaneous nodules when injected into the mice strains indicated below. They do not metastasize. Although these tumors will grow as subcutaneous nodules, the fact that they do not have a tendency to spread to other organs in the mouse will minimize animal suffering.

3. Species Identification and Rationale/Justification

The CB17 SCID mice will be used for xenografting in a human prostate cancer LNCaP. This strain of animal has been chosen because although the prostate cancer line LNCaP is not highly tumorigenic, when the cells are mixed with Matrigel the tumor-take following subcutaneous inoculation is very high (in the order of 95% at our Institution). The Syngeneic DD/S strain of mouse will be used for the *in vivo* tumor studies using the murine Shionogi hormone sensitive cancer line. The Shionogi line is an androgen sensitive carcinoma cell line that provides a model for steroid sensitive prostate cancer. The Shionogi line is actually a murine line and the choice of DD/S murine strain for these experiments are based on the fact that these mice are syngeneic with the Shionogi cell line. Experiments with the Shionogi tumors will allow confirmation of the efficacy of EB1089 & DHT in a second animal model of prostate cancer.

4. Numbers of Animals Required and Rationale/Justification

There are two types of experiments proposed with this animal use proposal. We plan to assay whether or not vitamin D3 analog EB1089 in combination with low physiologic doses of dihydrotestosterone (DHT), provided by the indwelling subcutaneous DHT pellet can inhibit the growth of prostate cancer in already established subcutaneous prostate cancer tumors that are formed following subcutaneous injection into immunodeficient mice. The ability of the EB1089 and DHT to inhibit tumor growth will be compared to controls of either EB1089 alone, low dose DHT alone, or a no-treatment arm. In the second project, we hope to ascertain whether the combination of EB1089 and DHT is better than either hormone alone or no-treatment in preventing tumor-take following subcutaneous inoculation of 10^6 human prostate cancer cells into immunodeficient mice.

In the trial to assay the inhibition of growth of established tumors, we will have 20 mice in each of 4 arms. Each of the mice will have a tumor cells injected subcutaneously into each flank, which will provide 40 tumors to be assayed in each of the four experimental arms. The four experimental arms will include no treatment, DHT pellet alone, DHT pellet with EB1089 and EB1089 alone. In the prevention trial, we will also use 20 animals on each of the four experimental arms. Thus 160 animals in total are requested. The total number of animals

required for this study, thus, will be approximately 200. Assays to verify the levels of testosterone are in the animals following the implantation of the DHT pellet, and castration must be carried out on approximately 20 animals. Similarly it is important to establish the dose of EB1089 following subcutaneous injection that does not cause hypercalcemia. This vitamin D3 analog does not cause hypercalcemia in humans but may cause it in experimental animals, if the dose exceeds a certain range. This range must be determined for this particular animal type.

5. Animal Research

The proposed animal research is also detailed in the Animal Use Protocol under the Methods section, point #7 Description of Project and Procedures in the appended Animal Use Protocol that has been approved at this Institution.

METHODS

A. Cell Lines. Human prostate cancer LNCaP cells, passage 40, are grown in RPMI 1640 with 5% fetal bovine serum (FBS). The Toronto subline of the transplantable SC-115 Shionogi mouse mammary carcinoma will be used.

B. In Vivo Tumour Growth. 6- to 8- week old male athymic nude mice (CrI:nu/nu(CD-1) BR) will be inoculated sc with 1×10^6 LNCaP cells suspended in 100 μ L Matrigel. 2×10^7 Shionogi cells will be injected into male DD/S mice. Tumours are measured twice weekly and their volumes calculated by the formula $L \times W \times H \times 0.5236$ (7). Blood samples for sequential DHT and PSA measurements will be obtained as previously described. Blood will be taken from the tail vein of the mice for serum calcium and for PSA and testosterone levels. Samples will not exceed 0.2 ml and there will be a waiting time of two weeks between sample takings.

C. Surgical Castration. Inject 0.20-0.25 ml of anesthesia IP. Wipe belly and lower abdomen with iodine swab. When mouse does not respond to pinching or squeezing his foot, make incision midline through skin. Cut through muscle wall being careful not to cut colon. Using forceps, pull left testicle and epididymal fat pad out of body. Tie three knots below testicle and fat pad with 3.0 chromic gut thread. Cut off testicle and fat pad above knots. Repeat for right testicle and epididymal fat pad. Stitch muscle wall by pulling a needle and 3.0 chromic gut thread through muscle on both sides of the incision and making three knots. Repeat at another point along the incision. Be careful not to pierce internal organs. Clamp skin together with 1 or 2 staples. The staples should be removed after 10-14 days. The post-doctoral fellow who will do these experiments is a urologic surgeon.

D. Histology and Immunohistochemistry. Tumors will be excised from sacrificed mice and processed for histological analysis as previously described. Deparaffinized and frozen sections will be incubated with monoclonal antibodies prepared against PSA, p27, and MIB1 (Ki-67), and digoxigenin-linked nucleotide and N-terminal deoxynucleotidyl transferase (Apotag, Oncor, Inc.) Frozen section will also be used for in situ hybridization to analyze changes in mRNA levels and distribution of these markers when appropriate.

E. Western Analysis. Tumour specimens harvested at various times pre- and post-castration and following treatment with DHT and EB1089 will be processed for cytoplasmic and nuclear

protein extraction and probed for antibodies against AR, p27, VDR, and actin as a loading control.

F. RNA Isolation and Northern Blot Analysis. Gene expression will be tested by Northern analyses using standard techniques.

G. Cell cycle profiles. The distribution of tumor cells in the different cell cycle phases will be monitored using tumor dissociation according to the Hedley method and routine preparation for FACS analysis.

EXPERIMENTAL DESIGN.

- A. Determination of physiologic DHT doses and normocalcemic doses of EB1089 in mice.** Several mice will be used to assay the serum levels of DHT following implantation of subcutaneous DHT pellets in castrate mice. We will use 12.5 mg subcutaneous pellets which should provide serum levels of 3-5 ng/ml in the mouse. Our goal is to approximately the serum levels of DHT in non-castrate mice without DHT pellets (eg 3-5 ng/ml serum). While EB1089 does not cause hypercalcemia in humans, it will be necessary to demonstrate that the doses recommended by the manufacturer, Leo, for use in mice (0.1-1.0 µg/kg/day) do not cause any negative side effects, including hypercalcemia in the mice. Both assays of DHT and calcium can be performed in the hospital's biochemistry department.
- B. Effects of DHT and EB1089 to suppress tumor formation.** We will test whether low dose DHT (delivered by sub-cutaneous pellet) and EB1089 can prevent tumor formation as follows. Mice will be injected at a maximum of 4 sites (max. vol. of 200 µl/site) sc with the respective tumor lines after a 2 week pre-treatment with DHT, DHT + EB1089, EB1089 alone or no treatment (10 mice each treatment category). Hormonal treatments will continue for the duration of the experiment. Tumor number and size and markers of proliferation and DNA analysis will be monitored at the termination of the experiment. The termination of the experiment will be defined as follows: Animals will be sacrificed 6 months after tumors arise or when the tumor reaches a mean subcutaneous flank tumor diameter of 17 mm, that is a volume exceeding (17 mm)³. Any animal bearing an ulcerated tumor will be sacrificed as soon as ulceration is detected.
- C. Effects of DHT and EB1089 to suppress established tumor growth.** DHT supplemented castrate mice will be injected at 4 sc sites per animal as above with either LNCaP or Shionogi cells and tumors allowed to establish over a 4 wk. period. We anticipate tumor sizes of <0.5 cm after 4 wks. After 4 weeks, hormonal treatment will begin. 10 Animals will be placed in each of 4 experimental arms: 1) no treatment controls 2) DHT 3) EB1089 4) both DHT and EB1089. Tumor size and parameters of proliferation and p27 levels will be monitored at 1, 2, 3, and 4 months or until the tumor reaches a mean subcutaneous flank tumor diameter of 17 mm whichever occurs first. Two mice will be sacrificed and tumors will be harvested prior to and 1, 2, 3 and 4 months after their appearance and divided into 3 portions: an unfixed portion will be used for FACS analysis; one portion will be fixed in 10% NBF and embedded in paraffin for morphological analysis; a third portion will be frozen at -80°C for Western blots and for RNA extraction and northern analyses. H&E and Apotag staining will be used to evaluate castration-induced cell death with characteristic apoptotic cells. Immunostaining using antibodies directed against the markers mentioned above will

be performed to evaluate qualitative and semi-quantitative (distribution and intensity) changes in respective protein levels following hormonal therapies (DHT and or EB1089). Internal reference slides will be used to normalize for variation in intensity of immunostaining between preps.

6. Anesthesia/Analgesia/Tranquilization

The animals will be anesthetized using intraperitoneal injection of a solution of 0.1 ml per 10 g mouse body weight of a mixture of ketamine plus xylazine solution prior to any surgical manipulation. The stock solution contains ketamine 75 mg plus xylazine 5mg (0.25 µl) in 4 ml of sterile water. Anesthetic will only be required in the experimental groups in which the animals undergo castration and implantation of the dihydrotestosterone pellets. No other requirement for anesthesia will be needed in the proposed experiments.

7. Study Endpoint

There are two projects and thus two endpoints in this proposal. In the first instance, we will ascertain whether DHT and EB1089 can suppress established tumor growth. Mice will be injected with mixture of Matrigel and LNCaP or Shionogi cells, and tumors will be allowed to establish over a four-week. We anticipate tumor sizes of < 0.5 cm diameter to arise during this period of time. Thereafter, animals will be divided into one of four experimental arms: no treatment, DHT only, EB1089, or DHT and EB1089. When the tumors reach a mean subcutaneous flank diameter of 17 mm or at four months following the onset of drug treatment, whichever occurs first. It is anticipated that the treatment arm of DHT plus EB1089 will provide greater inhibition of tumor growth in either DHT or EB1089 alone, and all of these treatments will be superior to no treatment in which the tumors are anticipated to grow continuously over the duration of the experiment.

In the prevention trial, the efficacy of EB1089 plus DHT in prevention of tumor take will be assayed by survey of treated animals for 6 months following the injection of tumor cells. If tumors do arise, they will be observed during treatment until they reach a mean subcutaneous diameter of 17 mm or for 6 months whichever occurs first. The animals will be euthanized at tumor diameters exceeding a mean of 17 mm.

8. Euthanasia or Final Disposition

The animals will be euthanized by cervical dislocation as indicated in point 14 of the Animal Use Protocol accompanied.

9. IACUC Approval

The animal research will be carried out in the Research Wing of the Sunnybrook & Women's College Health Sciences Centre. The protocol proposed has been approved by our Animal Use Protocol committee. The Animal Use Protocol duly completed has been approved.

A copy of our institutional Animal Welfare Assurance #A5176-01 from the US NIH Public Health Service is appended.

10. USDA Inspection Report

Because the studies will be undertaken in a Canadian University teaching hospital, we do not have US Department of Agriculture inspections. It is not possible to provide this report. However, the Statement of Compliance (Assurance) with Standards for Humane Care and Use of Laboratory Animals from our Institution has been approved by the NIH (documentation appended).

11. Qualifications

The animal facility at the Sunnybrook & Women's College Health Sciences Centre is approved by the Canadian Council on Animal Care. All of the personnel in the facility are appropriately trained. The individual carrying out the research project proposed, Dr. William Zhang, in the Cancer Biology Research Department is a postdoctoral fellow who is a urologic surgeon trained in China. This MD, PhD scientist has also undergone a one-day course that is mandatory for University of Toronto researcher prior to undertaking experimental protocols using animals. He also has considerable past experience in experiments with animals from his PhD training.

12. Accreditation

Please see the attached letter from Department of Health & Human Services, NIH and the Statement of Compliance with Standards for Humane Care and Use of Laboratory Animals by Foreign Institutions.

SIGNED ASSURANCES

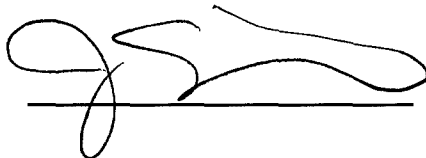
I assure that discomfort and injury to animals will be limited to that which is unavoidable in the conduct of scientifically valuable research and that analgesic, anesthetic, and tranquilizing drugs will be used where indicated and appropriate to minimize discomfort and pain to animals.

A handwritten signature in black ink, consisting of a large loop followed by a horizontal line and a small flourish.

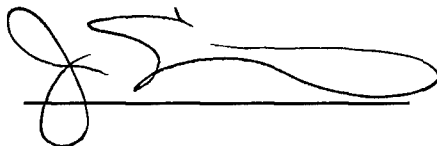
I assure that the animals authorized for use in this protocol will be used only in the activities, manner, and quantities herein, unless a deviation is specifically approved by my IACUC and the USAMRMC Animal Use Review Division.

A handwritten signature in black ink, consisting of a large loop followed by a horizontal line and a small flourish.


I accept full responsibility for the proper care and use of the animals during the conduct of research outlined in the proposal.

A handwritten signature in black ink, consisting of a large loop followed by a horizontal line and a small flourish.

I verify that I have made a reasonably good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

A handwritten signature in black ink, consisting of a large loop followed by a horizontal line and a small flourish.

I verify that the personnel performing the animal procedures/manipulations described in this protocol are technically competent in those procedures and have received training on the use of animals in research as required by the Animal Welfare Act of 1985.

A handwritten signature in black ink, consisting of a large loop followed by a horizontal line and a small flourish.

I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal and that the minimum number of animals needed for scientific validity are used.

A handwritten signature in black ink, consisting of a large loop followed by a horizontal line and a small flourish.



DEPARTMENT OF HEALTH & HUMAN SERVICES

PUBLIC HEALTH SERVICE
NATIONAL INSTITUTES OF HEALTH

FOR US POSTAL SERVICE DELIVERY:

Office for Protection from Research Risks
Division of Animal Welfare
6100 Executive Boulevard, Suite 3B01
MSC 7507
Rockville, Maryland 20892-7507

FOR EXPRESS MAIL:

Office for Protection from Research Risks
Division of Animal Welfare
6100 Executive Boulevard, Suite 3B01
Rockville, Maryland 20852
Telephone: (301) 496-7163 ext. 220
Fax: (301) 402-2803

July 31, 1996

Re: Animal Welfare Assurance #A5176-01

David Thomas
Director, Research Administration
Sunnybrook Health Science Centre
Reichmann Research Building, S-130
2075 Bayview Avenue
Toronto, Ontario M4N 3M5 Canada

Dear Mr. Thomas:

The Statement of Compliance (Assurance) with Standards for Humane Care and Use of Laboratory Animals that you recently submitted to the Office for Protection from Research Risks (OPRR), has been reviewed and accepted. A copy of the signed Assurance is enclosed.

Your Assurance has been assigned number #A5176-01 and became effective on 07/31/96. This Assurance supersedes any previously issued Assurance. The approval period is for five years, covers all Public Health Service (PHS) supported activities involving live vertebrate animals, and will expire on 06/30/01. Please include the Assurance number when corresponding with OPRR or with any funding component of the PHS.

OPRR must be advised of any changes in policies, procedures, or status of institutional sponsorship that would affect your Assurance.

Sincerely,

Denis J. Doyle
Chief, Assurance Branch
Division of Animal Welfare, OPRR

Enclosure

cc:
Dr. Jorge Filmus
Ms. Susan A. Bettendorf-Grant#5R21CA67132-02



STATEMENT OF COMPLIANCE WITH STANDARDS FOR HUMANE CARE AND
USE OF LABORATORY ANIMALS BY FOREIGN INSTITUTIONS

Sunnybrook Health Science Centre, hereinafter referred to as institution, hereby states that, in reference to the Public Health Service Policy of Humane Care and Use of Laboratory Animals, it will comply with laws, regulations, and policies regarding humane care and use of laboratory animals of the jurisdiction in which the research will be conducted.

I. Applicability

This statement is applicable to all research, research training, and biological testing activities involving live, vertebrate animals supported by the Public Health Service and conducted at this institution, or at another institution as a consequence of the subgranting or subcontracting of a PHS-supported activity by this institution.

II. Institutional Policy

This institution is guided by the *International Guiding Principles for Biomedical Research Involving Animals* developed by the Council for International Organizations of Medical Sciences. This institution will comply with all applicable provisions of the following laws, regulations, and policies governing the care and use of laboratory animals (LIST GOVERNING LAWS, REGULATIONS, AND POLICIES).

This institution acknowledges and accepts responsibility for the care and use of animals involved in activities covered by this Statement of Compliance. As partial fulfillment of this responsibility this institution shall make a reasonable effort to ensure that all individuals involved in the care and use of laboratory animals understand their individual and collective responsibilities for compliance with all applicable laws, regulations, and policies pertaining to animal care and use.

III. Institutional Endorsement and PHS Approval of Statement

Authorized Institutional Official

Name: David Thomas Title: Director, Research Administration

Address: Sunnybrook Health Science Centre, Reichmann Research Building, S-130
2075 Bayview Avenue, Toronto, ON M4N 3M5

Phone: 416-480-5720

Signature: [Signature]

Date: July 24, 1996

PHS Approving Official

Name: Denis J. Doyle Title: Chief, Assurance Branch

Address: Office for Protection from Research Risks, NIH

Division of Animal Welfare

6100 Excessive Boulevard, Suite 3B01, MSC 7507

Rockville, MD 20892-7507

Phone: 301-496-7163 Fax: 301-402-2803

Signature: [Signature]

Date: 7/31/96

Statement of Compliance # A5176-01 Effective Date 7/31/96 Expiration Date 6/30/01

2075 Bayview Avenue
North York, Ontario
Canada M4N 3M5

University
of Toronto

Principal Investigator (*last, first, middle*)

SLINGERLAND, JOYCE M.

BUDGET FOR ENTIRE PROPOSED PERIOD OF SUPPORT

BUDGET CATEGORY TOTALS*		INITIAL BUDGET PERIOD (FROM FORM PAGE 1)	ADDITIONAL YEARS OF SUPPORT REQUESTED				TOTAL
			2nd	3rd	4th	5th	
PERSONNEL		\$ 33,883	\$ 33,883				
FRINGE BENEFITS		5,829	5,829				
CONSULTANT COSTS							
MAJOR EQUIPMENT		1,811					
MATERIALS, SUPPLIES, AND CONSUMABLES		25,776	39,858				
TRAVEL COSTS		755	755				
RESEARCH-RELATED PATIENT COSTS							
OTHER EXPENSES		6,941	6,941				
SUBTOTAL DIRECT COSTS							
CONSORTIUM COSTS	DIRECT						
	INDIRECT						
TOTAL DIRECT COSTS		\$ 74,995	\$ 87,266				
TOTAL INDIRECT COSTS							
TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PERIOD OF SUPPORT					\$ 162,261		
TOTAL INDIRECT COSTS FOR ENTIRE PROPOSED PERIOD OF SUPPORT					\$		
TOTAL COSTS FOR THE ENTIRE PROPOSED PERIOD OF SUPPORT THIS AMOUNT SHOULD AGREE WITH THAT ENTERED ON THE COVER SHEET BOOKLET, ITEM #24					\$ 162,261		

* Itemize all budget categories for additional years on *Justification* page which follows.

addendum

Addendum to Year 2 budget justification				Amount \$Cdn	Amount \$US	Total \$US
Balance forward from original yr 2 budget submission						\$73,184
Additional Expenses for New Experiments Proposed:						
I. Animal Costs:						
1) mouse handling and housing						
	handling			\$151	\$104	
	housing (.82/cage/day)					
	(.82 x 100 cages x110 days)			\$9,020	\$6,218	
2) cost of animals (\$31 each)						
	(170 x \$31)			\$5,270	\$3,633	\$9,956
II. Equipment:						
	weigh scale			\$150	\$103	
	clipper			\$496	\$342	
	caliper			\$30	\$21	\$466
III Reagents:						
	DMT pellets \$2060US/100					
	placebo pellets \$1200US/100			\$4,729	\$3,260	
	matrigel			\$580	\$400	\$3,660
Sub-total additional expenses				\$20,426	\$14,082	
Total year two expenses						\$87,266
Institutional Cost Share						
Discipline Cost Share: Biological Sciences Discipline						
Secretarial & administrative support				\$23,072	\$15,906	
common equipment: photocopier, fax, FACScan, fluorescent microscope, slide projector, service contracts (photocopier/fax/FACScan), computers etc.				\$20,000	\$13,788	
communications charges including long distance				\$4,000	\$2,758	\$32,451
Institutional Overhead:						
(30% of direct costs excluding travel; see attached institutional overhead policy)						
this includes support for: utilities, common equipment, service contracts, building maintenance, 50% glass washing; support for core personnel etc.				\$36,210	\$24,963	
The cost of retrieval of primary prostate cancer tumor blocks and cutting of sections to provide unstained slides for p27 analysis will be covered by the Dept. of Pathology (cost-\$13 CDN/Case x 200 tumors = \$2,600)				\$2,600	\$1,792	\$26,755
Total Institutional Cost Share Years Two and Three				\$85,882		\$59,207
Currency exchange rate .689394 US = \$1 Cdn. as at February 29, 2000						

new # 323

Re: Dr. Joyce Slingerland "The Cell Cycle Inhibitor p27Kip1"
US Army - Prostate

1999 2000

joyce home

Tel 924-2725

7

ELEMENT	RATE	%	YR 1	YR 2	TOTAL
PERSONNEL					
Slingerland	n/a	na/	\$0	\$0	\$0
Battacharya	\$26,494	100.00%	\$26,494	\$26,494	\$52,988
Cariou	\$22,932	100.00%	\$7,389	\$7,389	\$14,778
Tshlias	n/a	100.00%	\$0	\$0	\$0
Total Direct Labor			\$33,883	\$33,883	\$67,766
FRINGE BENE					
Slingerland			\$0	\$0	\$0
Battacharya			\$5,829	\$5,829	\$11,658
Cariou			\$0	\$0	\$0
Tshlias			\$0	\$0	\$0
Total Fringe Benefits			\$5,829	\$5,829	\$11,658
TOTAL DIRECT LABOR			\$39,712	\$39,712	\$79,424
CONSULTANTS			\$0	\$0	\$0
EQUIPMENT			\$1,811	\$0	\$1,811
SUPPLIES			\$25,776	\$25,776	\$51,552
TRAVEL			\$755	\$755	\$1,510
PATIENT COSTS			\$0	\$0	\$0
OTHER EXPENSES			\$6,941	\$6,941	\$13,882
SUBTOTAL			\$74,995	\$73,184	\$148,179
SUBGRANT DIRECTS			\$0	\$0	\$0
SUBGRANT INDIRECTS			\$0	\$0	\$0
TOTAL DIRECTS			\$74,995	\$73,184	\$148,179
TOTAL INDIRECTS			\$0	\$0	\$0
TOTAL COST			\$74,995	\$73,184	\$148,179

institutional cost share
Government share

58,152 \$58,152 \$116,304
\$74,995 \$73,184 \$148,179
\$264,483

US Army

Ms. Scassero:

I concur with the above budget.

and to start Jan 1/99

Judy Tong

Judy Tong
Grants Coordinator
Sunnybrook Research

11/15
11/16
11/17

Objective: To define the policy and procedures for the distribution of the 30% overhead charge on all contract research at Sunnybrook Health Science Centre.

Preamble: Research in Canada has developed as a partnership between the funding agencies (i.e. MRC, NCI, H & S, etc.) and the host institutions (i.e. hospitals and universities). A typical pattern for this partnership is for each dollar of institutional money to be matched by at least two dollars from the funding agencies. This is not the situation in the U.S.

Corporate funding for medical research in Canada has followed this same funding pattern resulting in institutions directly subsidizing contract research from the corporate sector (pharmaceuticals, bio-technology, medical devices). This pattern of subsidy is now widely recognized and all academic institutions are beginning to collect overheads to cover the indirect costs of corporate research. Sunnybrook Health Science Centre has imposed a 30% overhead effective August 1, 1993.

The policy will be reconsidered in June, 1996 should there be a negative impact on the volume of research.

Policy: The 30% overhead on contract research will be used to ensure increased protected time for clinician researchers, for the strategic development of new clinician researchers, and to offset the infrastructure cost of support of clinical research.

Procedure:

The 30% overhead will be distributed as follows:

1. In the Department of Medicine: 20% will be given to the Research Committee of the Department of Medicine for protection of time and clinician researcher development provided it is matched with Departmental funds to the Department of Medicine Research Committee.
2. In the Department of Surgery: 10% will be given to the academic plan of the Division provided it is matched with Divisional funds for protected time and clinician research development, and 10% to the individual research project.
3. In Research Programs: 10% will be given to the Program Director for researcher salary support and 10% to the individual research project.
4. In other Medical and Professional departments and units: 10% will be given to a collective body (to be determined) provided it is matched by funds from the collective group for protected time and professional researcher development, and 10% to the individual research project.

The remaining 10% will be kept by Research Administration to offset infrastructure costs, such as biostatistics, ethics, grants administration, animal facilities subsidies, E-2 clinician researcher support, etc.

Overhead Charge on all Contract Research
at Sunnybrook Health Science Centre

Research in Canada has developed as a partnership between the funding agencies (i.e. MRC, NCI, H & S, etc.) and the host institutions (i.e. hospitals and universities). A typical pattern for this partnership is for each dollar of institutional money to be matched by at least two dollars from the funding agencies.

Corporate funding for medical research in Canada has followed a funding pattern whereby institutions directly subsidize contract research from the corporate sector (pharmaceuticals, bio-technology, medical devices). This pattern of subsidy is now widely recognized and all academic institutions are beginning to collect overheads to cover the indirect costs of corporate research. These overhead costs include heat, hydro, environmental services, regulatory compliance: biomedical and fiduciary, ethics, human resources, etc. Sunnybrook Health Science Centre has imposed a 30% overhead effective August 1, 1993.



SUNNYBROOK HEALTH SCIENCE CENTRE - RESEARCH

COLLECTION AND DISTRIBUTION OF OVERHEAD ON CONTRACTUAL RESEARCH

Payments for contractual research are often contingent on work progress, patient enrolments, completion of milestones, as opposed to fixed instalments based on a budget. These factors may cause the actual income to differ from the anticipated budget. For this reason, the collection AND distribution of overhead monies will be handled at the time the payments are received.

Vice-President, Research
Room S-132, S-Wing
Reichmann Research Bldg.
Tel. (416) 480-4293
FAX (416) 480-5894

PROCEDURE

FOR SUNNYBROOK-HELD ACCOUNTS

Prior to commencement of project, the "Notice for Contractual Research" is completed and sent to Research Administration office (S104) along with a copy of the contract agreement. The Notice serves as a control sheet that all parties concerned are informed.

On contracts where equipment and travel budgets are included, allowances will be made in the calculation as required, since overhead is NOT levied on these items.

Each time a payment is received from Sponsor, 10% will be coded directly to the Department/Division/Program cost centre, 10% to Res. Admin. and 10% will be returned to the investigator's account. A copy of the "Notice" form will then be sent to the Department and to the P.I. for their record whenever this transaction takes place.

FOR SHUTC-HELD ACCOUNTS

It is the responsibility of the P.I. to declare the overhead collectible for funds that are not held at Sunnybrook. The investigator should contact Research Administration and their Dept/Div head to advise on when and how to recover the overhead component. The attached form may be used for this purpose, but indicate "SHUTC ACCOUNT" on top of form.

Any exception to the rule in overhead policies has to obtain prior approval from the Vice-President of Research.

APR/95 RESEARCH ADMINISTRATION

2075 Bayview Avenue
North York, Ontario
Canada, M4N 3M5

University
of Toronto



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

13 Feb 02

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218

SUBJECT: Request Change in Distribution Statements

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statements for Accession Document Numbers listed at enclosure be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Request for Change in Distribution Statements

Accession Document Numbers

ADB261483

ADB267652

ADB257443

ADB257429

ADB266030

ADB257323

ADB244279

ADB269814

ADB232291